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Examination of Blood for Tumor Cells[1, 2]

AVERY A. SANDBERG *and* GEORGE E. MOORE,[3]
Departments of Medicine and Surgery, Roswell Park Memorial Institute, Buffalo, New York

It has been long recognized that the spread of malignancy to distant sites in the body probably involves transport of tumor cells either through the lymphatic system or the blood. Nevertheless, the demonstration of tumor cells in the blood, or for that matter in the lymphatic circulation, has been the subject of only a few sporadic reports through the years. In a recent paper, Engell (*1*) reviewed earlier studies concerned with the occurrence of cancer cells in the peripheral blood and in the blood of veins draining tumor sites.

In this country, several investigators have reported tumor cells in the blood or perfusates draining tumor sites, particularly those associated with carcinomas of the large bowel (*2*, *3*). The presence of tumor cells in venous blood of vessels adjacent to a tumor are of special interest. Studies reporting this phenomenon have been interpreted as indicating the necessity of ligating all possible venous channels before the tumor is manipulated preparatory to its removal.

In this communication we wish to present briefly a simple method for the examination of blood specimens for the presence of tumor cells. In addition, some preliminary data concerning our findings to date will be presented. Since many of the patients were selected for study because of the advanced state of their disease, associated diagnostic problems, or inclusion in a chemotherapy program, the findings may not be representative of an unselected group of patients with malignancy.

Only the initial blood sample obtained from each patient is included in the statistics, even though subsequent samples were positive in a few instances.

Methods

The following method was found to give the best results for the concentration of tumor cells in the blood. Five ml. of blood was carefully mixed with 80 mg. of bovine fibrinogen[4] dissolved in 2 ml. of water con-

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[2] Assisted by grants from the Dorothy H. and Lewis Rosenstiel Foundation, the Elsa D. Jameson Memoria Fund, and the American Cancer Society, Inc.
[3] We wish to thank Miss Jean Rae Schubarg for invaluable technical assistance.
[4] We wish to thank Dr. J. B. Lesh, of Armour & Co., for generous supplies of bovine fibrinogen.

taining 1 mg. of heparin. In some instances, 3 to 4 ml. of saline was added to reduce the concentration of red blood cells and thus accelerate their sedimentation. After sedimentation for 15 to 20 minutes, the plasma layer was carefully removed with a syringe and needle and centrifuged at 1000 r.p.m. for 5 minutes. The plasma was then decanted and smears were prepared from the concentrated mixture of cells and plasma that remained. Three slides were routinely stained by either the Wright or May-Grünwald-Giemsa technique. Two additional smears were treated by the Papanicolaou method for independent examination. Wherever possible direct imprints of the cut surface of the tumors were prepared for comparison with the cellular morphology seen in the blood smears. Other methods for the separation and concentration of tumor cells were tried but proved to be unsatisfactory. Lysing the red blood cells with water or hypotonic solutions resulted in poor recovery of tumor cells from blood to which Ehrlich ascites-tumor cells had been added. In addition, the preparations were not satisfactory as smears, for there was considerable distortion of morphology. Examination of the buffy coat for tumor cells following centrifugation of the whole blood yielded poor results, because many of the tumor cells settled with the red blood cells and could not be recovered.

Three slides prepared from a single buffy-coat sample were first examined by a trained technologist and all slides with suspected tumor cells were re-examined by one of the authors. All of the negative slides were rechecked by the other author. In general, each slide can be scanned under low power in approximately 15 minutes. The recognition of tumor cells in blood is not simple. Their recognition requires not only intimate knowledge of the cells normally occurring in the blood or bone marrow but, in addition, acquaintance with the appearance of tumor cells and their differentiation from atypical cells (endothelial and connective-tissue cells, etc.). The following routine was established. A technician, well versed in the examination of blood, screened all slides and recorded all those with tumor or atypical cells. The slides were then examined by one of the authors, whose recognition of the tumor cells was based on his experience with tumor cells in bone-marrow specimens and on comparison of the tumor cells with the cells obtained from an imprint of the tumor or with tumor cells seen in the fluids of the body cavities. It is apparent that an all-embracing definition of tumor cells cannot be given. In general, the tumor cells are larger than the largest cell of the blood, their cytoplasm is often slate blue and "foamy" in appearance and may contain occasionally fine azurophilic granules. The nucleus of the tumor cells is usually very large and contains several large nucleoli of light-blue color. When these cells are present in clumps they can be easily identified.

In a few cases, cells that could not be identified as tumor cells but that were not normal blood elements were found; they were labeled "atypical." It should be emphasized that, in general, there was a tendency to underestimate the number of positive slides, since in some smears the tumor

cells may resemble immature cells normally present in peripheral blood; these were listed as "negative."

Successive patients from the Chemotherapy Service and patients from 3 surgical services form the basis of this report. Incidental control specimens resulted from changes of the initial clinical diagnosis. Such patients are probably more valuable controls than healthy individuals, since severely ill patients are more apt to have immature and atypical cells in the blood. In addition, blood specimens from 10 normal subjects were examined.

Results and Comments

Preliminary results on a small group of patients are shown in table 1. In 105 patients having advanced adenocarcinomas with metastases, tumor cells were present in the peripheral blood in 45 and absent in 60. Four patients with lymphosarcoma had negative slides and 1 was positive. In 5 cases abnormal cells were observed and listed as atypical. Patients with tumors considered to be most active biologically also had a larger percentage of positive blood specimens. For example, there were 4 positive and 3 negative samples from patients with carcinoma of the lung; and in 2 patients with malignant melanoma smears were positive.

TABLE 1.—*Preliminary results*

Sources	Regional vein		Peripheral blood*	
	Total	Positive	Total	Positive
Gastrointestinal malignancies	25	14	49	18
Breast	8	4	36	18
Lung	3	1	7	4
Sarcoma	1	1	13	6
Ear, nose, and throat	7	1	9	2
Other: kidney, thyroid, pancreas, malignant melanoma, testes, etc	4	1	15	7
Benign lesions	7	0	3	0
TOTAL	55	22	132	55

*A majority of these patients were from the Chemotherapy Service and had advanced disease, but they were not terminal patients.

The second part of the study involved securing blood samples during celiotomy from veins draining the sites of malignancy. These were collected both before and after surgical manipulation necessary for removal of the tumor. Of 12 such patients with negative premanipulation samples, tumor cells were found in only 1 sample following manipulation. Operative-site specimens from patients with gastric carcinoma were almost all positive, even from those with resectable lesions. In most instances, tumor-imprint preparations from the operative specimens were available for a comparison with the blood smears.

No tumor cells were found in the blood of the normal subjects or of patients with nonmalignant diseases.

The blood smears of representative cases are shown in figures 1 to 12.

Further study will be necessary to substantiate these preliminary findings and clarify the sampling errors involved in the examination. Our preliminary observations parallel those of Engell (*1*), who found an equal number of tumor cells in the regional veins both before and after manipulation of the tumor in the abdominal cavity. This observation may not be true for malignancy of the lung.

With this technique it may be possible to record additional evidence of the effectiveness of cancer chemotherapy by serial examination of the blood for tumor cells. For example, recently a patient with advanced gastric cancer with many tumor cells in the peripheral blood was treated by us with actinomycin D, following which striking objective and subjective improvement was observed. Concomitantly, the blood samples became free of tumor cells. The duration of this remission was only 2 weeks and, as measurable growth of metastases again occurred, the peripheral blood samples again contained cancer cells. Similar observations have been made in 2 patients with advanced breast and gastric cancers, respectively, treated with triethylenethiophosphoramide.

It should be emphasized that the examination of blood smears for the presence of tumor cells requires considerable knowledge of the morphology of the normal cellular elements present in the blood. Cells that may confuse the inexperienced observer, such as abnormal, large, or immature lymphocytes and plasma cells, may be found, especially in blood draining the gastrointestinal tract, and may be mistaken for malignant cells. One has the impression that blood from cancer patients has a larger number of plasma cells, which, if immature, are often large and dark-staining, contain large nucleoli, and may undergo mitosis. In addition, the examiner must have considerable experience in differentiating other cells that may be confused with cancer cells, *e.g.*, atypical myelocytes and endothelial or connective-tissue cells. Nevertheless, on the basis of our experience in the examination of tumor imprints stained with Wright or May-Grünwald-Giemsa stains, we have found that a competent technologist can become proficient at the detection of tumor or atypical cells in the blood under the guidance of a hematologist.

Summary

A procedure for the detection of cancer cells in blood has been described that is simple and yields excellent preservation of cell morphology. The presence of tumor cells in the peripheral blood of patients with advanced malignancy and in regional veins draining tumor sites is not uncommon. This procedure should be of interest in studying the biological nature of various tumors, routes of dissemination, mechanisms for the destruction of tumor cells, and, possibly, as an additional method of evaluating anticancer agents.

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(*1*) ENGELL, H. C.: Cancer cells in the circulating blood. Acta chir. scandinav., supplement 201, 1955.

(*2*) POOL, E. H., and DUNLOP, G. R.: Cancer cells in the blood stream. Am. J. Cancer 21: 99–102, 1934.

(*3*) FISHER, E. R., and TURNBULL, R. B., JR.: The cytologic demonstration and significance of tumor cells in the mesenteric venous blood in patients with colorectal carcinoma. Surg., Gynec. & Obst. 100: 102–108, 1955.

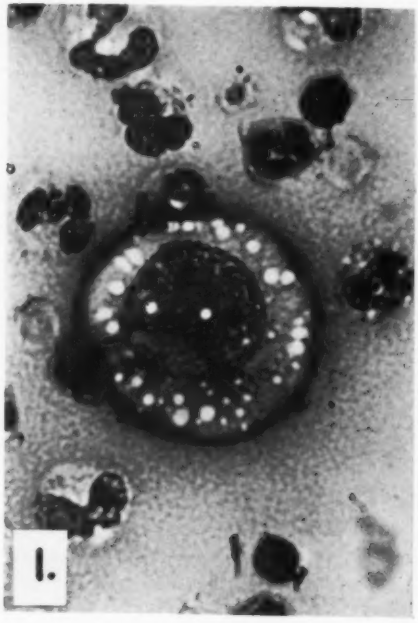
PLATE 1

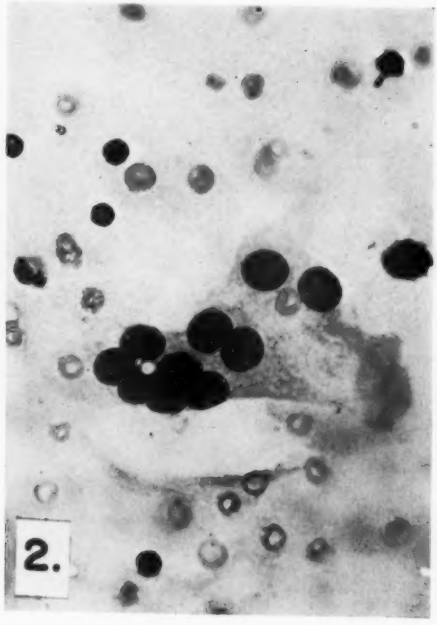
FIGURE 1.—Patient J. L., 53-year-old male, carcinoma of right lung. Specimen obtained from vein draining tumor site following manipulation of the tumor. Cell resembled those seen in tumor imprint. × 1200

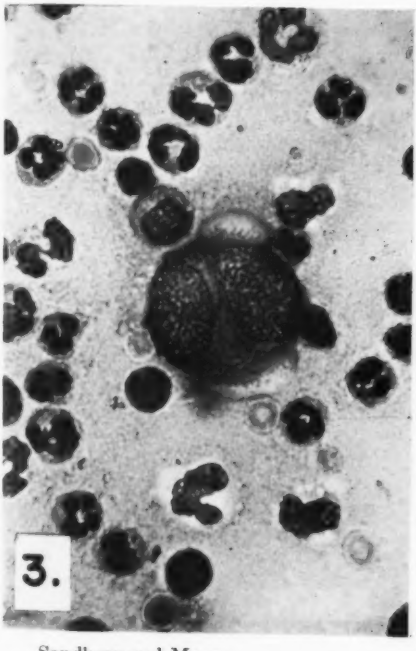
FIGURE 2.—Patient E. A., 55-year-old female, carcinoma of right breast. The cells are probably of endothelial origin and were not called tumor cells. × 1200

FIGURE 3.—Patient J. L. of figure 1. Tumor cell is similar to those seen in tumor imprint. × 1200

FIGURE 4.—Patient J. L. of figure 1. Tumor cell with prominent nucleoli. × 1200







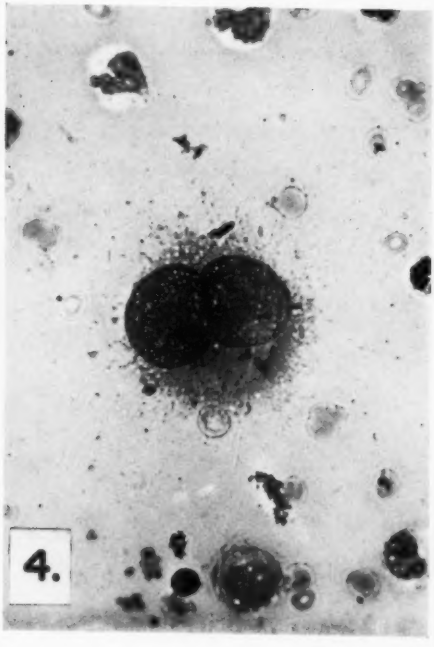


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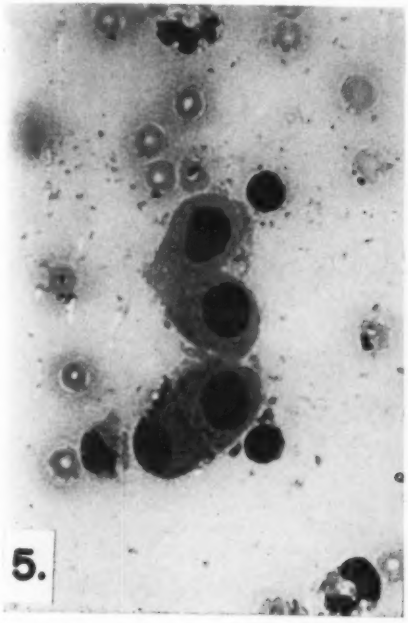
PLATE 2

FIGURE 5.—Patient D. K., 20-year-old male, carcinoma of right kidney. Tumor cells in peripheral blood. Similar cells, occurring in large clumps, were seen in the bone marrow. × 1200

FIGURE 6.—Patient J. V., 72-year-old male, carcinoma of the colon. Clump of tumor cells present in vein draining tumor site. Blood was obtained following manipulation of the tumor. Similar cells were seen in tumor imprint. × 1200

FIGURE 7.—Patient A. B., 67-year-old female, carcinoma of uterine fundus. Clump of tumor cells present in vein draining tumor site. Similar cells were seen in ascitic fluid. × 1200

FIGURE 8.—Patient R. A., 58-year-old male, carcinoma of left lung. Tumor cells in blood draining tumor site. × 1200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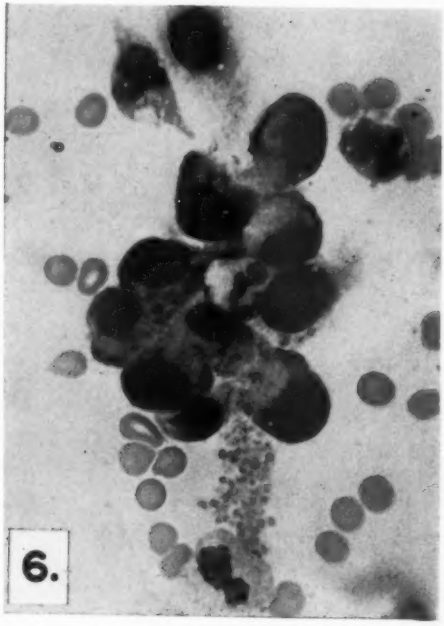
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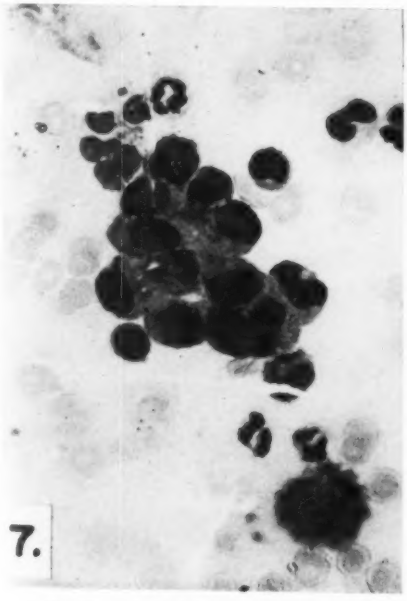
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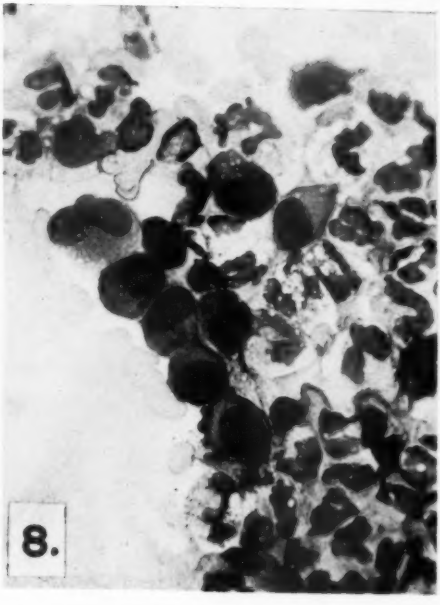
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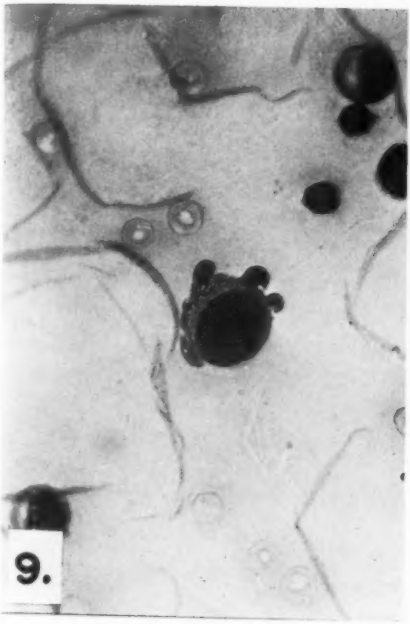
PLATE 3

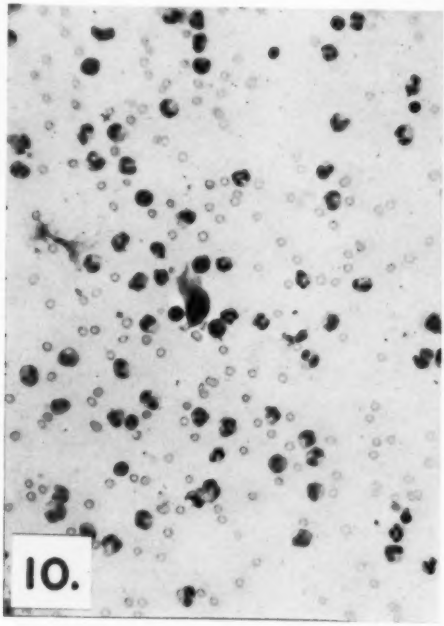
FIGURE 9.—Patient M. K., 37-year-old male, malignant melanoma. Cell in peripheral blood resembled malignant-melanoma cells present in bone marrow and in imprints of skin tumors. × 1200

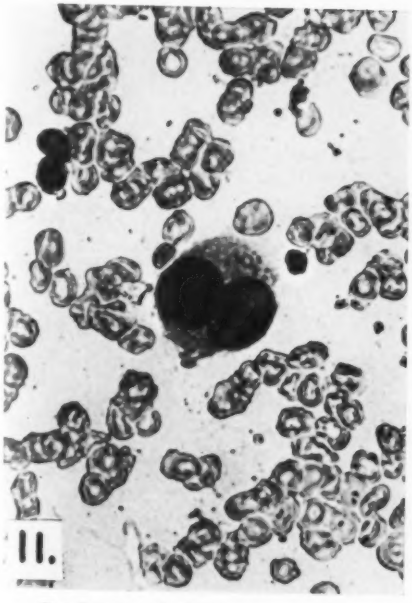
FIGURE 10.—Patient A. D., 63-year-old female, carcinoma of stomach. Cells in peripheral blood obtained immediately following gastrectomy. Cell was classified as a probable tumor cell. × 200

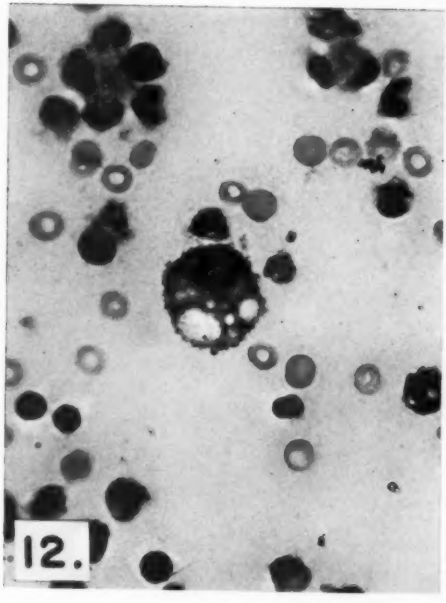
FIGURE 11.—Patient L. A., 63-year-old female, carcinoma of breast. Tumor cells in peripheral blood. *Note* prominent nucleoli and large size of cell. × 1200

FIGURE 12.—Patient S. M., 28-year-old male, malignant schwannoma. Tumor cell in vein draining tumor site before manipulation. × 1200









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Quantitative Studies on the Multiplication of Neoplastic Cells *in Vivo*. IV. Influence of Doubled Chromosome Number on Growth Rate and Final Population Size [1,2]

THEODORE S. HAUSCHKA, SARAH T. GRINNELL, LÁSZLÓ RÉVÉSZ, *and* GEORGE KLEIN, *Roswell Park Memorial Institute, Buffalo, New York, and Wallenberg Laboratory, Institute for Cell Research and Genetics, Karolinska Institutet, Stockholm, Sweden*

Perhaps the major experimental advantage of ascites over solid tumors is the precision of growth measurements based on the total number of free tumor cells in the peritoneal cavity. The variables of necrosis and host stroma are minimized. Initial cell dose can be corrected for the fluctuating admixture of nonmalignant host elements. Sequential determinations of cell number, total packed-cell volume (ascitocrit), mitotic index, and mean cell generation time are reproducible from one test to the next with comparatively small standard deviations.

In 3 earlier papers of the present series (*1–3*), the growth characteristics of several mouse ascites tumors have been considered in terms of the above parameters. These investigations included Ehrlich carcinoma (probably not related to the Ehrlich lines used in the present study), sarcoma MC1M, Gardner lymphosarcoma 6C3HED, Dalton thymoma, and Gorer lymphoma EL4. Growth curves for these and other ascites tumors (*4–6*) exhibit some broad similarities but also consistent differences that are especially pronounced when one compares the invasive lymphomas as a group with the more slowly infiltrating anaplastic carcinomas and sarcomas.

The rapid-growth phase of some ascitic tumors appears as an exponential multiplication of free tumor cells with no appreciable lag. Various types of equations can, however, be fitted more or less meaningfully to portions of sigmoid curves. For example, the Ehrlich ascites carcinoma did not grow at a constant exponential rate (*1*); because of continuous lengthening of mean cell generation time it followed a linear cube-root increase of cell number with time.

Little information exists on the complex nutritional, toxic, immunologic, and "homeostatic" graft-host relationships that determine the shape of growth. Cell-number asymptotes attained by various tumors in mice of comparable age and weight differ from case to case, depending on growth speed, isoantigenicity, invasiveness, and other properties (*5, 7*). Each neoplasm may, indeed, be limited to a characteristic maximal proto-

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[2] Supported in part by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and the Swedish Cancer Society.

plasmic mass, the nutrilite contributions to which—rather than the toxic products or anatomical inroads of which—are lethal for its host.

Such questions pertinent to growth cannot be approached through comparisons of tumors differing in origin, histology, histocompatibility, and cell function; they demand measurements on genetically related sublines derived from the same neoplasm and dissimilar in only a single variable.

In the present analysis, this major variable is the chromosome number. What happens to growth when the nuclear constitution—and concomitantly the individual cell volume—is doubled? Which is most influential as a check on cell multiplication: total cell number attained, total protoplasmic mass, or total parasitic cell surface exposed to the host? Developmental genetics has taught us that the genes of any given karyotype differ widely in their dosage effects. Some are and some are not cumulative. Hence, doubling of the chromosome number, accompanied by a compensatory cytoplasmic increase, may change the synthetic potential per unit volume of protoplasm. In many plant and animal tissues polyploidization does not alter nucleo-cytoplasmic ratios (*8*). This homeostasis of cell mass, closely geared to size constancy of organ and organism (*e.g.*, heteroploid amphibians), allows no *a priori* conclusions as to the intrinsic growth capacity of enlarged cells.

Doubling the volume of a sphere results in a surface increase by a factor of only 1.6. If the smaller surface/volume ratio retards molecular traffic across the plasma membrane, then, other factors being equal, tetraploid cells should be outpaced by corresponding diploids.

Availability of near-diploid ascites tumors and their polyploid derivatives has brought such speculations under experimental attack. Growth studies were carried out with 2 cytogenetically related line pairs of the Ehrlich carcinoma, the hyperdiploid *ELD* and its hypertetraploid offshoot *ELT*. Thus it was possible to compare tumor cells of genotype A and mass 1 with sister cells of genotype 2A and mass 2. Since the findings at our 2 laboratories (Buffalo and Stockholm) confirmed earlier preliminary data (*9*) and were mutually supportive, they are pooled in this joint publication.

Materials and Methods

Tumors

The hyperdiploid Ehrlich ascites tumor and its cytologically almost identical offshoot, known under the synonym of "Landschütz Sarcoma I" (*10–12*), were received in 1953 from Dr. H. Lettré and Dr. C. Landschütz, respectively. Both lines are nonspecific in their histocompatibility and are here referred to as *ELD*. Their uncertain descent via Loewenthal and Jahn's original ascites tumor (*13*) from one of Ehrlich's many spontaneous mouse carcinomas is considered to be traceable as far back as 1896 (*10*).

The aneuploid modal chromosome number of 45–46 (diploid = 40 in the mouse) and 5 clear "marker" chromosomes have persisted essentially

unchanged (*14*) since 1952, when Bayreuther (*10*) first described the *ELD* idiogram. The structurally distinguishable chromosomes include 3 "minutes," smaller than the shortest normal mouse chromosome, 1 element with a secondary constriction, called "A," and the metacentric "B" unit (fig. 1). Up to 6 percent of polyploid cells arising through endomitotic processes such as "endoreduplication" were present in *ELD* samplings from our routine serial transfers (fig. 2).

After intraperitoneal injection of low immunizing doses of *ELD* cells into Ha/ICR Swiss mice, Kaziwara (*12*) was able to select out and concentrate the apparently less antigenic (*15, 16*) hypertetraploid duplications of the stem-cell karyotype. His so-called "EL 88" subline had a modal chromosome number of 90–92. A similarly doubled cell population was isolated after we inoculated 8 mice with 20×10^6 irradiated *ELD*/Stockholm cells (4000 r *in vitro* in an N_2 atmosphere). Many of the irradiated cells were probably nonviable; thus the low-cell-dose conditions of Kaziwara's selection experiments were reproduced. Three mice were sufficiently immunized to remain entirely tumor-free for 6 months; 4 died within 2 months with ascites and abdominal infiltrations; in 1 mouse a solid intra-abdominal growth was palpable after 4 months. A suspension was made from this tumor in Krebs-Ringer-phosphate and about 10^6 cells were injected intraperitoneally into 10 additional mice, all of which developed hypertetraploid ascites tumors.

The 2 polyploid sublines used in the present growth studies are as comparable as their 2 *ELD* parent tumors and are henceforth called *ELT*. It should be emphasized that the latter are cytogenetically more homogeneous and have at least 10 more chromosomes per stem cell than the near-tetraploid Ehrlich ascites (*16–18*) used in earlier growth experiments (*1, 6*).

If one works with initial cell doses below 7×10^5 per mouse, the histocompatibility differences between *ELD* and *ELT* cells favor concentrative immunoselection of the naturally present 3 to 5 percent *ELT* minority from a predominantly hyperdiploid cell population. At cell doses above 1×10^6, immune reactions have no detectable effect on growth phenomena. However, the *ELT* populations are not stable in routine serial passage; they tend to revert gradually toward predominance of the hyperdiploid cell minority (*12, 19*), unless immunoselection is continuously encouraged through the simple expedient of low cell dose. Another way around the dilemma of ploidy drift is viable frozen storage at −70° C., followed by thawing and 1 shuttle passage prior to growth tests. The Stockholm *ELT* line was permanently stabilized by isolation of single cells, whereafter it retained its hypertetraploid character both cytologically and by DNA content.

The pathophysiology of the *ELD* and *ELT* sublines was grossly similar. The ascites was rather blood-free; abdominal distension was uniform; mean exudate volume 9 days after injection of 20×10^6 cells was 3.4 cc. for *ELD* and 3.0 cc. for *ELT*; invasiveness into the viscera was not extensive; the fibrous clots of cellular debris appearing in the fluid at the

terminal stages of growth were of comparable dimension. Virulence, as measured by survival time of over 200 matched mice after inocula of 20×10^6 cells, was almost identical (16.42 ± 0.19 days for *ELD*; 17.50 ± 0.19 days for *ELT*).

Thus, *chromosome constitution* and cell volume are the only major variables in our Ehrlich ascites lines. Our determinations for these properties are summarized in table 1, together with pertinent data published by others (*11*, *19*). The chromosomes were counted in numerous well spread metaphases on aceto-orcein squash preparations (*20*) made throughout the course of our study. These numerical findings agree with earlier data (*10–12*) and with DNA determinations (*19*). The values in table 1 reveal an approximate 2:1 relationship between *ELT* and *ELD* with regard to chromosomal, DNA, and cell-volume characteristics.

DNA content of 0.2 to 0.4 ml. of undiluted ascites was determined in 2 aliquots of each sample by Schneider's method (*21*). The values were expressed as average amounts per tumor cell.

Average tumor-cell volume was obtained by dividing the volume of cellular sediment (determined by the hematocrit technique) by the cell concentration in a corresponding aliquot for each individual mouse. The results were then expressed in cubic microns.

Determination of Total Number of Free Tumor Cells in Peritoneal Fluid

Rinse technique.—This technique is a modification of a previously published procedure (*1*). At appropriate intervals during tumor growth matched cage lots of 8 to 10 mice, all of which had received identical inocula from the same ascites pool, were killed by cervical fracture. They were then pinned individually on drainboards standing in large glass funnels, a T-shaped incision was cut through the abdominal wall, and all the ascites fluid, plus 40 cc. of rinsing Ringer solution, was collected into an Erlenmeyer flask. The total tumor-cell number was calculated for each mouse from cell counts made in 2 separate bright-line hemocytometers by 2 observers.

Dye-dilution technique.—Details of this method were published elsewhere, and good quantitative agreement with the rinsing procedure was

TABLE 1.—*Nuclear properties and cell volumes of 4 Ehrlich ascites lines used in present growth studies*

Property	Tumor line			
	ELD/Buffalo	*ELT*/Buffalo	*ELD*/Stockholm	*ELT*/Stockholm
Modal chromosome number...	45–46	90–92	46 (*11*)	~92
Percent polyploid cells.....	<6%	>86%	<3%	~85%
Mean cell volume..	1099μ^3 (*19*)	2026μ^3 (*19*)	1368μ^3 ± 47	2238μ^3 ± 67
DNA per cell.....	10.0×10^{-9} mg. ± 0.3 (*19*)	17.5×10^{-9} mg. ± 1.5 (*19*)	11.1×10^{-9} mg. ± 0.5	20.6×10^{-9} mg. ± 1.1

obtained (*2*). The bromsulfalein method was used in the growth measurements of the Stockholm *ELD* and *ELT*. Growth curves of both tumor lines were determined in 2 separate experiments, each comprising 30 to 40 mice inoculated from a common pool with 20×10^6 tumor cells (corrected for normal host cells with the aid of Papanicolaou smears). Groups of these mice were then sacrificed for cell counting at standard intervals. The geometric mean was calculated for each lot of total cell numbers, consisting of 4 individual determinations from 2 separate experiments.

Irradiation

To determine mitotic duration by the method of Widner *et al.* (*22*), 2 mice bearing Stockholm *ELD* and 2 with *ELT* were irradiated with 1250 r (165 kv., 1 Al filter, intensity 80 r per minute) on the 5th day of tumor growth. Every 15 minutes during a period of 1 hour after irradiation, 0.1 ml. samples of ascites fluid were tapped from each mouse with a fine glass capillary, and a Papanicolaou smear was prepared from each tapping. On each slide 2,000 tumor cells were surveyed for mitotic index, *i.e.*, the number of cells in all mitotic stages per 100 tumor cells.

Statistical Methods

Statistical parameters were determined for individual growth curves and for the important differences between *ELD* and *ELT*. For the determination of the confidence interval for the cell-number ratios in table 3 we are greatly indebted to P. R. Sheehe, Dept. of Statistics and Epidemiology, Roswell Park Memorial Institute, Buffalo, New York.

Mice

The Buffalo *ELD* ascites and its *ELT* branch were propagated and their growth was measured in female Ha/ICR Swiss mice, 2 to 3 months old and weighing 23 to 28 gm. These animals were reared at our Philadelphia ICR and Buffalo RPMI laboratories. For serial tumor passage, 0.2 ml. undiluted ascites containing about 35×10^6 cells was transferred intraperitoneally at 10-day intervals. The growth data in text-figures 1 and 6 and tables 2 to 4 are based on tests carried out in Philadelphia and Buffalo with Ha/ICR Swiss mice.

The Stockholm *ELD* and corresponding *ELT* were similarly passaged and studied in male heterozygous albino mice, 2 to 3 months old and ranging from 18 to 22 gm. in weight. The mice were bred on the laboratory premises at the Karolinska Institute and yielded the Stockholm data in text-figures 2 to 5. All the experimental animals received a standard pellet diet and drinking water *ad libitum*.

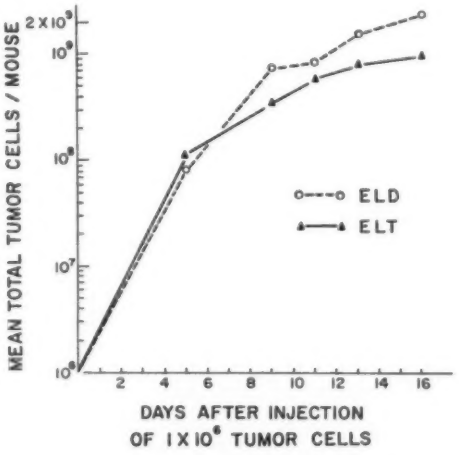
Results

Tumor-Cell Number

According to Kaziwara (*12*), immunoselection of polyploid *ELT* from the predominantly hyperdiploid *ELD* Ehrlich ascites operates after

"small" starting inocula—from 500,000 cells downward. We have found an initial dose of 1 million tumor cells per mouse just large enough to avoid differential effects of the host's immune reactions on the growth of the 2 tumor lines.

Text-figure 1 shows the increase in total number of free tumor cells for 2 groups totaling 100 Swiss female mice. The animals were separated into 10 matched cage lots of 10 each and were injected intraperitoneally with 1 million *ELD* or *ELT* cells, respectively. Mean total cell numbers per mouse were determined from individually counted groups of 10 by the quantitative rinsing method.



TEXT-FIGURE 1.—Growth of the hyperdiploid Ehrlich ascites *ELD* and its hypertetraploid subline, *ELT*. Each point represents mean total tumor-cell number for a group of 10 adult Swiss female mice.

The 2 ploidy classes of cells multiply at nearly the same rate during the early, rapid phase of growth. Presumably because of the larger polyploid cell volume, the growth environment becomes suboptimal for *ELT* sooner than for the hyperdiploid tumor. The averages of 2.47×10^9 *ELD* and 1.04×10^9 *ELT* cells reached on day 16, *i.e.*, shortly before death, can be considered maximal for mice weighing 23 gm.

Table 2 contains statistical details for the growth curves shown in text-figure 1. On day 5, the total tumor-cell numbers for *ELD* and *ELT* do not differ significantly ($P > 0.20$), whereas the numerical difference becomes highly significant from day 9 onward ($P < 0.01$) and approximates a ratio of 2.

Essentially similar results obtained with a starting dose of 20 million Ehrlich cells are shown in text-figure 2 for the Stockholm line of *ELD* and its hypertetraploid derivative. Each point represents the mean value for 4 male mice. These weighed about 5 gm. less than the animals in the preceding series, hence they sustained the growth of decidedly

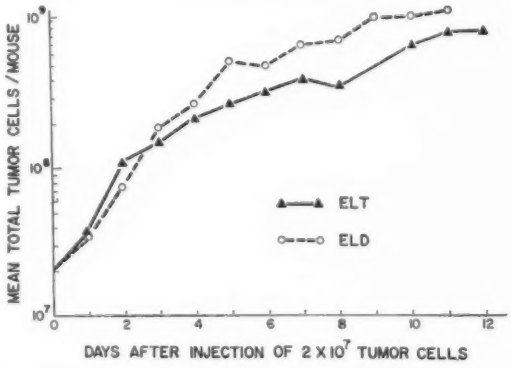
TABLE 2.—*Mean total tumor-cell numbers per mouse after injection of 1 × 10^6 cells of the hyperdiploid Ehrlich ascites,* ELD, *and its hypertetraploid subline,* ELT*

Tumor line	Day after injection of 10^6 cells	Mean total† cells per mouse × 10^6	Standard error × 10^6	Standard deviation × 10^6	Ratio *ELD* : *ELT*
ELD	5	82. 2	24	76	0. 73
ELT		111. 7	9	27	
ELD	9	786. 0	71	214	2. 16
ELT		365. 5	34	110	
ELD	11	891. 0	40	127	1. 44
ELT		619. 9	47	151	
ELD	13	1640. 9	235	753	2. 00
ELT		819. 6	177	566	
ELD	16	2469. 6	267	800	2. 38
ELT		1038. 1	197	555	

*These data correspond with the points on the 2 growth curves of text-figure 1.
† Each mean is based on 10 mice.

smaller total tumor-cell numbers and died earlier. Between the 5th and 11th day after inoculation the *ELD* : *ELT* ratio averaged 1.7.

The reproducibility of constant "saturation" cell number during the terminal growth phase after different initial cell doses was remarkable (table 3). From the 4 separate comparisons of *ELD* and *ELT* in matched groups of mice one may calculate a mean ratio of *ELD* maximum : *ELT* maximum per mouse. This observed ratio averaged 1.9 when the starting cell *number* was the same for *ELD* and *ELT* (table 3, lines 1–4) and 2.3 when the initial cell *mass* was adjusted to the same level (table 3, lines 5–8). The true value can be placed between 1.50 and 2.94, with a confidence level of 95 percent (P for departure of the data from a theoretical 2.0 expectation >0.60). Five additional measurements of mean total cell number at "saturation time" (2 *ELD* tests on 18 mice;



TEXT-FIGURE 2. Growth curves for the Stockholm line of the hyperdiploid Ehrlich ascites tumor *ELD* and its hypertetraploid branch, *ELT*. Each point represents mean total tumor-cell number per mouse, based on 4 mice.

3 nonmatched *ELT* tests on 24 mice) gave average values of 1901×10^6 for *ELD* and 943×10^6 for *ELT*, *i.e.*, a ratio of 2.0, which supports the significance of the observations in table 3.

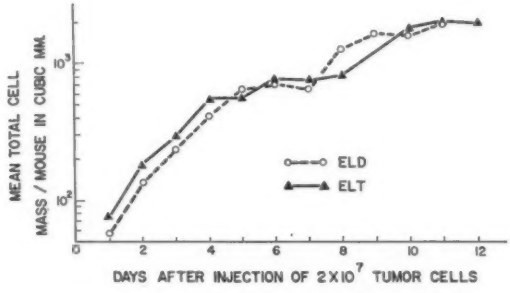
TABLE 3.—*Reproducibility of maximum mean total tumor-cell number per mouse ("saturation mass") in 4 separate comparisons of the hyperdiploid Ehrlich ascites,* ELD, *with its hypertetraploid derivative,* ELT

Tumor line	Tumor cells injected per mouse	Day	Surviving mice	Mean total tumor cells per survivor $\times 10^6$	Cell-number ratio *ELD* : *ELT*
ELD	1×10^6	16	10/10	2470	2.4
ELT	1×10^6		10/10	1038	
ELD	20×10^6	17	10/20	2124	1.5
ELT	20×10^6		10/20	1343	
ELD	35×10^6	14	7/8	2055	2.1
ELT	20×10^6		8/8	990	
ELD	35×10^6	13	8/8	2367	2.5
ELT	20×10^6		8/8	942	

Tumor-Cell Mass

The mean killing cell mass can be estimated roughly from ascites contents of the survivors at 50 percent mortality time. The product of mean tumor-cell volume, in cubic microns (*see* table 1, line 3), and total cell number per mouse on day 17, when half of 40 mice had died (*see* table 3, lines 3 and 4), was 2.94 ml. of wet packed *ELD* cells and 2.86 ml. of wet packed *ELT* cells, or a little above one tenth of the starting mouse weight (26 gm.) in this series. These determinations are probably biased, since the survivors are apt to be individuals supporting less than lethal tumor-cell mass.

In text-figure 3 the tumor-mass increment for the Stockholm *ELD* and *ELT* after a starting dose of 20 million cells per mouse is plotted over a 12-day course. Until day 4, the total mass of the hypertetraploid



TEXT-FIGURE 3.—Growth of the hyperdiploid Stockholm Ehrlich ascites *ELD* and its hypertetraploid derivative, *ELT*. Determinations of mean total packed-cell volume per mouse were made from the same samples plotted in text-figure 2.

line is in the lead. Thereafter, the 2 mass curves converge toward nearly identical plateaus at "saturation time."

Throughout this period, cell concentration per *ml.* of ascitic fluid was fairly constant: 231.6 ± 11.5 million *ELD* cells and 163.9 ± 9.2 million *ELT* cells. With the aid of corresponding mean cell volumes (*see* table 1, columns 4 and 5) one may calculate total protoplasmic mass per *ml.* of exudate, which was 367 × 10^9 μ^3 for *ELT* and 317 × 10^9 μ^3 for *ELD*.

Average Cell Generation Time

The increasing cell generation times for 3 arbitrary growth intervals are compared in table 4. After a similar initial doubling rate of *ELD* and *ELT* from day 0 to 5, the hypertetraploid cells decelerate first and slow down gradually toward a terminal doubling time of 160 hours, as against 81 hours for the hyperdiploid line. It may be assumed that these determinations are fairly representative averages for the cell population; cellular viability showed no appreciable decline during the progressive growth of several ascites tumors (*1*, *2*), including the present material. Infiltration of solid intra-abdominal tissues was present but of limited and comparable extent in both *ELD* and *ELT*.

TABLE 4.—*Mean cell-doubling times* calculated from the growth curves of the hyperdiploid* ELD *tumor and its hypertetraploid derivative,* ELT

Tumor line	Initial cell dose per mouse	Growth period (*hours*)		
		Day 0–5	Day 5–11	Day 11–16
Hyperdiploid Ehrlich *ELD*.....	1 × 10^6	18.7	41.0	81.4
Hypertetraploid Ehrlich *ELT*.....	1 × 10^6	17.3	59.0	160.0

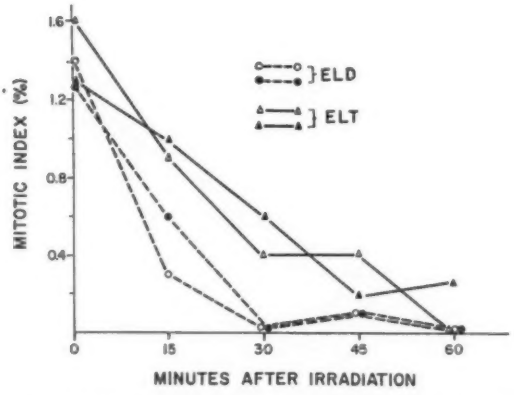
*These determinations correspond to the data in text-figure 1 and table 1. They were calculated with the standard formula $g=\frac{T_2-T_1}{\log N_2-\log N_1}\log 2$, wherein T_2-T_1 is the growth time, N_1 the starting cell number per mouse, and N_2 the total cell number recovered at the end of the observation period.

Mitotic Duration

Average mitotic-duration time (late prophase to telophase) is, according to Widner *et al.* (*22*), the time necessary for the complete disappearance of mitoses in an X-rayed population. It would appear from the mitotic index of samples taken every 15 minutes after irradiation with 1250 r (text-fig. 4) that an *ELD* mitosis requires about half the time (30 minutes) necessary for an *ELT* division (60 minutes). The latter time is in accord with earlier measurements for 2 other near-tetraploid ascites tumors (*1*). The slight residue of mitoses in the *ELD* samples at 45 minutes may be attributed to the approximately 5 percent minority of spontaneously arising polyploid elements that are always present in diploid tumors.

Cell Growth in Absence of Mitosis

After the complete disappearance of mitoses following X irradiation administered during the early rapid-growth phase, the tumor cells are now unable to perform cytokinesis; however, they continue to build proto-



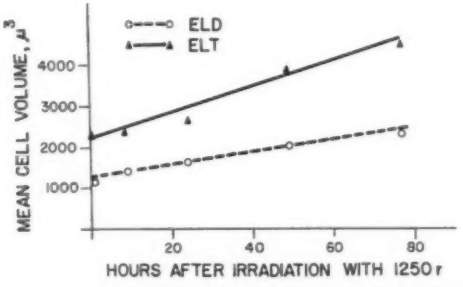
TEXT-FIGURE 4.—Disappearance of mitoses in the hyperdiploid Stockholm Ehrlich ascites *ELD* and its hypertetraploid branch, *ELT*, after irradiation with 1250 r. The points were obtained in 2 separate comparisons and represent single samples obtained by puncturing 4 mice individually at regular intervals.

plasm at an unchanged rate, as previously shown by Klein and Forssberg (*23*). This affords an experimental opportunity for comparing the inherent synthetic potential of *ELD* and *ELT* (text-fig. 5).

While the percentage increase of cell volume up to 76 hours after irradiation was the same for the 2 Ehrlich lines, their actual volume increase differed by a factor of 2. Calculations from the slope of the regression lines show that the average volume of the hyperdiploid cells increases 1.37 percent per hour and that of the hypertetraploids, 1.38 percent per hour. In absolute values this means the synthesis of 15.3 μ^3 of new cell mass per hour per *ELD* cell and 31.6 μ^3 per hour per *ELT* cell.

Immunologic Advantage of ELT *over* ELD

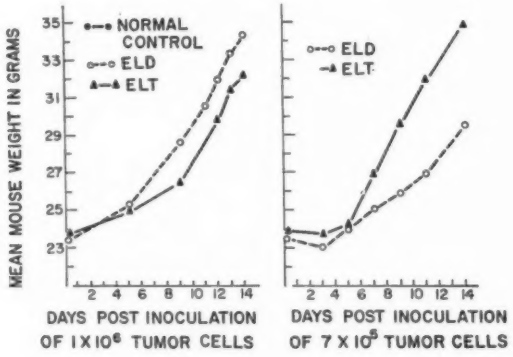
Although the hyperdiploid Ehrlich ascites may be classified as a "nonspecific" tumor with regard to its histocompatibility in a wide range of mouse genotypes, it is, nevertheless, possible to immunize mice with low



TEXT-FIGURE 5.—Increase of tumor-cell volume of irradiated hyperdiploid Stockholm Ehrlich ascites *ELD* and its hypertetraploid branch, *ELT*, in the absence of mitoses.

cell doses. All the preceding growth measurements were obtained after "large" initial cell doses between 1 and 35 million cells, which do not give the host time to produce inhibitory amounts of antibody. Kaziwara (*12*) achieved immunoselection of *ELT* cells after doses below 500,000 *ELD* cells per mouse, and this result was repeated by us and others (*19*) with the same tumor line and with immunizing doses (*24*) of irradiated Stockholm *ELD* cells.

Text-figure 6 is an indirect demonstration of the immunologic advantages of *ELT* over *ELD*, obtained at the upper threshold of an *ELD* immunizing dose (7×10^5 cells), beyond which (1×10^6 cells) the differential immune response of the host is ineffectual.



TEXT-FIGURE 6.—Increases in mean mouse weight after inoculation with 1×10^6 tumor cells (lowest level of "*overwhelming*" dose) or 7×10^5 tumor cells (upper dose level permitting development of a mild immune response against *ELD* but not against *ELT* cells). Each of the 4 curves is based on 20 animals. The weight increase during 2 weeks for normal Ha/ICR Swiss mice of comparable age is less than 2 gm.

The increases in host weight are due largely to ascites accumulation, which past work (*1*, *25*) has shown to be correlated with total tumor-cell number. The relatively constant cell concentration per ml. of ascitic fluid (*ELD* = 232×10^6 cells/ml.; *ELT* = 164×10^6 cells/ml.) permits conservative translation of net weight gain (corrected for 1.6 gm. normal mouse growth over the same period) into total cell number attained on day 14. After the "immunizing" dose of 7×10^5 cells, *ELD* growth is significantly retarded (about 0.9×10^9 cells/mouse on day 14) and *ELT* outgrows it (about 1.5×10^9 cells/mouse on day 14). The antigenic advantage of *ELT* is canceled out after "nonimmunizing" doses above 1 million cells per mouse. Under the latter conditions *ELD* reaches a 14-day total of 2.2×10^9 cells per mouse and *ELT* 1.1×10^9 cells per mouse. These estimates, calculated from the weight rise shown in text-figure 6, are in good agreement with actual cell counts, presented in table 3.

Growth Advantages of ELD *over* ELT

During serial transplantation of established *ELT* lines, which contain a residue of 5 to 15 percent hyperdiploid cells, care must be taken to use small enough cell doses for transfer; otherwise the *ELD* cells gradually regain numerical predominance in the population. This trend was noted by Kaziwara (*12*) and was guarded against as a source of potential error in enzymatic comparisons of *ELD* with *ELT* (*19*).

It is interesting that immunologic advantage does not bestow a growth advantage above a certain dose level. Below this level, the hyperdiploid cells seem handicapped by their greater antigenicity. Above this level they have 3 principal competitive advantages over *ELT*: As the growth rate decelerates, their cell generation time becomes progressively shorter than that of *ELT*. The *ELD* mitotic-duration time (which amounts to a small fraction of total cell generation time) is half as long, and the cell surface/volume ratio is 20 percent greater. At "saturation time," 2×10^9 *ELD* cells confront the host mouse with a total neoplastic surface of about 1.03 square meters, while the total surface of 1×10^9 *ELT* cells measured about 0.76 square meters (calculated from cell volumes in table 1 with the formula

$$S = \sqrt[3]{\frac{4}{3}\pi .27\ V^2}, \text{ where } S = \text{surface and } V = \text{volume}).$$

These differences apparently lead to eventual predominance of hyperdiploid elements in a mixed population, when large cell doses above 1×10^6 cells per mouse are routinely given during serial passage. However, during the 2-week course of an individual growth experiment, which, in the present studies, always begins with rather homogeneous *ELT* (>86% polyploid) and ELD (>94% hyperdiploid) ascites, the above factors cannot interfere with the quantitative appraisal of the major variable, *i.e.*, effect of the chromosome constitution on growth potential.

Discussion

The shape of a neoplastic growth curve is the complex product of competitive synthesis, anatomic impairment, nutritional depletion, host defenses, etc. Each tumor has, so to speak, its own sigmoidal style, which is as characteristic as its histology.

Growth patterns published for various ascites tumors (*1–6*, *24*) were distinct in several respects: They were either continuously ascending until death of the host or reached a plateau long before death. Initial lag was shown by some, but not by other, tumors; still others declined significantly during the terminal phase. The major portion of the growth curve fitted either exponential or cube-root increment. Individual cell volumes and cell viability were constant throughout growth or showed a gradual decline. These differences emphasize the important role of tumor genotype in resistance to variable immune responses, adverse conditions due to crowding, accumulation of waste products, and, above all, competitive utilization of available nutrients. Here, one may con-

sider the possible existence of rate-limiting anatomic barriers between the ascites-tumor cells and the host, such as the walls of the peritoneal blood vessels and the peritoneum itself. Are the tumor cells competing in a given limited space for more or less constant amounts of limiting nutrients? Are these quantities independent of the host's daily food intake and determined by the amount of diffusion into the peritoneal exudate?

The energy and nitrogen metabolism of tumor cells makes them efficient "N-traps" (*26*, *27*); they are capable of concentrative amino acid uptake (*28*) and cause excess lipide loss unrelated to the host's dietary intake (*29*). The proteins of a growing tumor are not released as fuel to the host, even during starvation. Since tumor growth in a starved animal is almost as rapid as in a well-fed host, LePage *et al.* (*30*) concluded that neoplastic protein metabolism is essentially a "one-way passage."

However, a puzzling inability of Ehrlich "Landschütz" *ELD* cells to utilize abundant free nucleotides, proteins, and other potential RNA precursors in the ascites fluid, despite a 50 percent decrease in intracellular ribonucleic acids, was observed by Ledoux and Revell (*31*) in a cytochemical growth study. This decrease in RNA was accompanied by a slower growth rate, which was reversible after serial transplantation.

Very little is known concerning tumor growth products that inhibit host metabolism. For example, the inhibitors of liver catalase, which are elaborated by the Ehrlich ascites (*6*) and many other neoplasms, are not tumor-specific, since liver catalase activity can be depressed significantly by injection of normal homogenized mouse spleen (*32*). On the other hand, tumor-specific production of a humoral factor which decreased diphosphopyridine nucleotide synthesis by normal tissues was recently reported (*33*). This factor lowered DPN synthesis by mouse blood to about 50 percent of normal within 1 hour after intramuscular tumor implantation.

In view of these multiple influences on the course of tumor growth and host response it is imperative that a growth comparison such as the present one be conducted with material of the same basic genetic constitution. With regard to their genome, the *ELT* cells represent exact duplications of the *ELD* karyotype. Hence, the observed dissimilarities between the 2 growth patterns can be ascribed to differences in chromosome ploidy.

The salient growth features may be recapitulated as follows: The intrinsic rates of protoplasmic synthesis per unit cell volume are the same for the hyperdiploid and hypertetraploid Ehrlich lines. During the initial stages of unimpeded growth, the 2 ascites tumors have the same cell-doubling time, until about 2×10^8 *ELD* or 1×10^8 *ELT* cells have accumulated. The cell mass attained by the 2 tumors at the end of the rapid-growth phase (which of necessity is shorter for *ELT*) is the same. Decelerating growth then continues toward a terminal maximum tumor mass, which is independent of the initial cell dose over the tested range of 1×10^6 to 35×10^6. This terminal neoplastic mass is also the same

for the 2 tumor lines, being composed of about 2×10^9 *ELD* or 1×10^9 *ELT* cells; translated into wet packed-cell mass this means about 2.2 ml. of either tumor or about one tenth of the host's body weight. Thus, regardless of cell-volume differences, the growth potential of different ploidy classes, which are different multiples of the same basic genotype, appears limited to a constant end mass, at least in the ascites form.

The observed *ELT*:*ELD* ratios for the major growth parameters were:

Modal chromosome number	2.0
Cell volume (Buffalo)	1.9
Cell volume (Stockholm)	1.7
Cell surface	~1.4
DNA per cell	1.9
Terminal cell number	~0.5
Terminal cell mass	~1.0
Cell generation time (during rapid growth)	0.9
Cell generation time (during terminal growth)	2.0
Mitotic duration	~2.0
Concentration of free cells per ml. ascitic fluid	0.7
Concentration of cellular mass per ml. fluid	1.2

It is suggested by these findings that the limiting factor for the multiplication of cells of the Ehrlich ascites lines is the total mass of the population rather than the cell number as such. In accordance with its 20 percent larger surface/volume ratio, the hyperdiploid line evokes the accumulation of slightly more ascitic exudate per unit cell mass than its polyploid counterpart. The agreement between the ratios for average DNA content per cell and average cell volumes indicates that the close correlation between chromosome ploidy and cell volume in various biological systems (*8*, *34*, *35*) also holds true for cancer tissue.

The Ehrlich ascites growth determinations may serve as idealized, but nonetheless instructive, models for the complex course of acute autonomous neoplasia. They furnish a norm for judging experimental growth inhibition of the widely used Ehrlich lines by antimetabolites, cytotoxic agents (*36*), irradiation (*24*), etc., and provide physiologically more revealing reference data than would the weight of a solid tumor, host weight, or host survival time.

Summary

The influence of chromosome constitution on growth characteristics was analyzed in 2 cytogenetically related pairs of Ehrlich ascites tumors. The 2 hyperdiploid lines (*ELD*) and the corresponding hypertetraploid derivatives (*ELT*) are alike in their pathophysiology and virulence. They differ by a factor of 2 in their nuclear content and cell volume.

Growth of these lines was compared with regard to the following parameters: total cell number per mouse at different intervals, total tumor-cell mass for the same growth periods, cell-doubling time, mitotic duration, mass increase in irradiated cells in the absence of mitosis, maximum cell number, and maximum neoplastic mass synthesized per mouse.

During the early-rapid growth phase *ELD* and *ELT* cells multiply at the same rate, until intercellular competition and/or exhaustion of the

host's nutritional and other resources begins to retard further synthesis of neoplastic substance. This happens earlier for *ELT* than for *ELD* after inoculation of the same number of tumor cells. The maximum lethal end mass attained was approximately 2.2 ml. of tumor protoplasm per mouse or one tenth of mouse weight. The corresponding asymptotes of cell number were about 2×10^9 hyperdiploid *ELD* cells or 1×10^9 hypertetraploid *ELT* cells. In smaller mice these numbers were proportionately reduced. Thus, total neoplastic mass, rather than cell surface or cell number as such, is the principle determinant of growth limits in the free-cell populations of Ehrlich ascites tumors.

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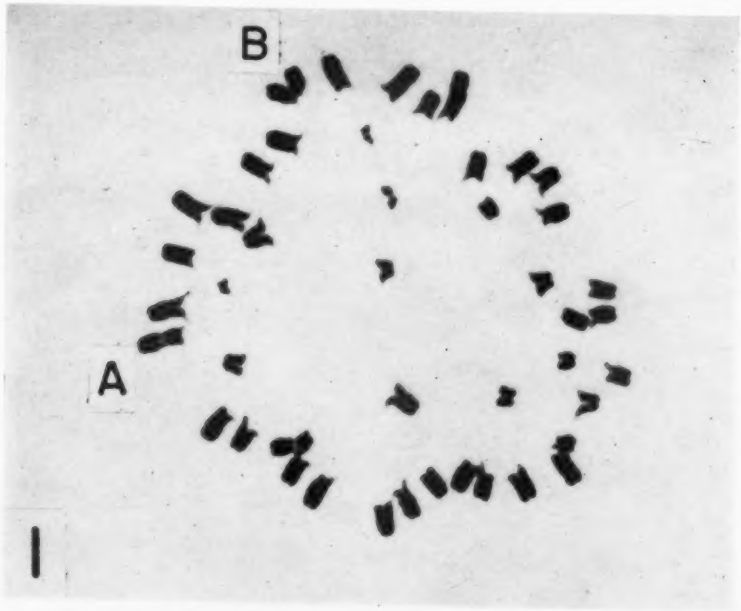
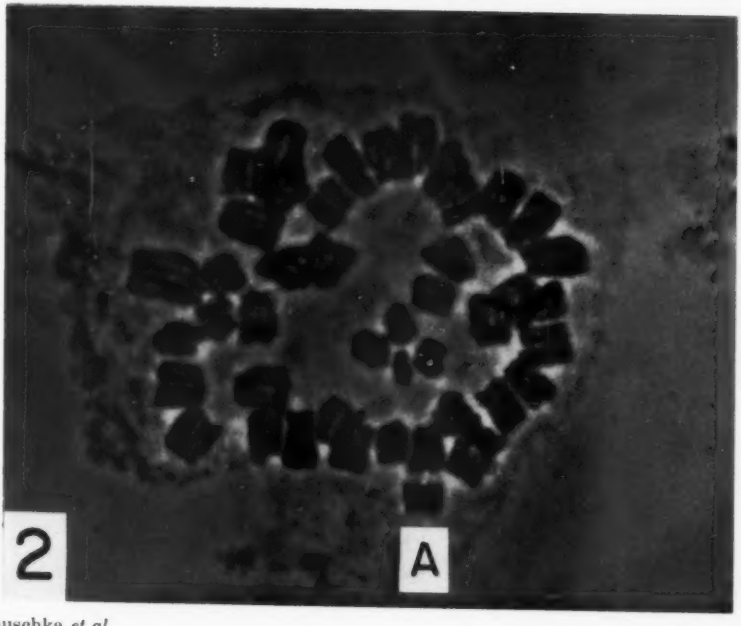
PLATE

PLATE 4

FIGURE 1.—Hyperdiploid metaphase with 46 chromosomes from an aceto-orcein squash of the Stockholm *ELD* Ehrlich ascites tumor, photographed by D. Hungerford. The "A" chromosome with its secondary constriction *below 9 o'clock*, the metacentric "B" *at 11 o'clock*, and the 3 "minutes" *at left* and *upper center*, smaller than the shortest normal mouse chromosomes, characterize the stem line of this tumor. × 1650

FIGURE 2.—"Endoreduplication" metaphase with 45 four-stranded chromosomes from an aceto-orcein squash of the Buffalo *ELD* Ehrlich ascites tumor, photographed by T. S. Hauschka. This rare stage is an endomitotic mechanism preceding polyploidization and results in hypertetraploid *ELT* cells. The "A" chromosome with its heterochromatic region is clearly visible *at 6 o'clock*, the metacentric "B" probably overlaps with 2 other chromosomes *at 10 o'clock;* 1 "minute" may be seen at *lower center*, the other 2 "minutes" are superimposed above longer chromosomes *near 2 and 5 o'clock*, respectively. × 2000





Hauschka *et al.*

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The Occurrence of Skin Tumors in Untreated Albino Mice from Dealer's Stock [1, 2]

PHILIPPE SHUBIK, KAY SPENCER, *and* GIUSEPPE DELLA PORTA, *Division of Oncology, The Chicago Medical School, Chicago, Illinois*

A particular sensitivity to tumor induction by croton oil has recently been reported. It was found that skin tumors develop in a large proportion of albino mice treated with croton oil alone, whereas similar treatment of other albino mice or of CA F_1 or C3H mice did not result in tumor induction (*1*, *2*). The report of tumor induction in these albino mice is at variance with many reports in the literature that mice develop either no tumors, or very few, when treated with croton oil only (*3–6*). It is, however, in keeping with a report by Roe (*7*) that a large proportion of mice developed papillomas and carcinomas when treated with croton oil alone. The present study is concerned with the albino mice reported to possess this singular responsiveness to croton oil. Mice obtained from the dealer have been studied and compared with their F_2 generation, bred in this department.

Experimental

A group of 100 stock albino mice, 4 to 6 weeks old, were received from Mr. Arthur Sutter, Springfield, Missouri. These mice were maintained under close observation, and every effort was made to exclude contact with possible carcinogenic contamination. The mice were housed in new plastic cages and kept initially in an isolation room and then in a mouse room. In neither of these air-conditioned rooms was any treatment performed or any chemical carcinogen kept. The mice were fed Rockland mouse diet and given water *ad libitum*.

During an initial 2-week isolation period, 40 of the 100 mice developed a severe enteric infection and died or had to be sacrificed. At 10 weeks of age the remaining 60 mice were mated for breeding. At 5 months of age 75 F_1 generation mice had been produced, and breeding was discontinued. Several of the breeding mice had died by this time, mostly because of fighting among the males, which resulted in severe ulceration and infection on the skin of the back. The 41 remaining mice of the original

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[2] This investigation was supported by research grant CS-9212 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and research grant ENV-7 from the American Cancer Society, Inc.

shipment were then separated by sex and placed in 3 cages containing, respectively, 12 and 16 females and 13 males. One week subsequent to the separation of these mice it was observed that 1 of the females, in the cage of 12, had developed a sessile papilloma on the face. It was then decided to observe this cage of females and the cage of males without any treatment. The females in the cage of 16 were subjected to mechanical irritation with a wire brush on 2 occasions, 8 weeks apart; no other treatment was given.

The female mouse that developed the first papilloma subsequently developed a total of 6 papillomas and 3 sebaceous adenomas on various parts of the body. One of the papillomas regressed and 2 were transformed into squamous-cell carcinomas, as diagnosed histologically. Of the remaining 11 mice in this cage, 5 subsequently developed tumors—7 papillomas and 1 sebaceous adenoma—between the 5th and 7th months of age. One mouse had 4 tumors, and the others had single tumors.

Only 2 of the 13 male mice maintained without treatment developed tumors at 8 months of age. One mouse developed 5 papillomas, none of which regressed, and the other, 3 papillomas, all of which regressed.

Two of the 16 females subjected to mechanical irritation developed tumors between the 6th and the 8th month of age. One mouse developed 2 papillomas and a squamous-cell carcinoma, and the other, a single papilloma in the region of the anus.

Fifty of the 75 F_1 mice were used for breeding, and the other 25 mice were discarded. By the time the F_1 mice were 7 months of age, 106 mice of the F_2 generation had been produced. The F_1 breeders that had not developed any tumors by this time were then discarded.

Fifty females and 26 males of the F_2 generation were then set aside for observation. At the time this report was written these mice were 10 months old, and none had yet developed a tumor. At 4 months of age, 15 females and 15 males of the F_2 generation were given twice-weekly treatments of 5 percent croton oil [3] in mineral oil.[4] After 20 weeks, 1 male had developed a single papilloma in the painted area, which has since regressed. No other tumors have been noted in this group after 23 weeks of treatment.[5]

As a control, 52 male and 40 female Swiss mice, originally from Bar Harbor stock and bred in this department during the past 6 years, were maintained under conditions similar to those for the Sutter mice. At the 12th month, there were 32 male and 40 female survivors with no skin tumors. At the 15th month, 1 male developed a small papilloma on the back.

Conclusions

It has been found that a commercially bred strain of mice (Sutter) develops a high incidence of skin tumors without treatment. It is notable

[3] Oleum crotonis (BP), Boots Chemists, Nottingham, England.
[4] Superla 34, Standard Oil of Indiana.
[5] This experiment is now at the 38th week and no tumors have developed.

that these tumors consist not only of papillomas and squamous-cell carcinomas but also of appreciable numbers of sebaceous adenomas. Sebaceous adenomas, papillomas, and carcinomas have recently been reported as occurring in hairless mice, and their origin has been attributed to contact with a wood preservative used in their cages (*8*). It appears likely that the origin of the skin tumors in the Sutter mice may be attributed to the same cause. The F_2 generation of these same mice did not develop tumors either spontaneously or when painted repeatedly with croton oil. Rusch *et al.* (*1*) and Boutwell *et al.* (*2*) performed the majority of their experiments in mice obtained directly from the dealer. The activity of croton oil in these animals can, perhaps, be attributed to previous initiation. However, Boutwell *et al.* (*2*) found that croton oil also induced tumors in the F_1 generation of these mice bred in their laboratory.

Certain general considerations emerge from this study. Although one shipment of mice has been found to yield a relatively high incidence of skin tumors without treatment, other shipments are apparently different. Among those previously reported only 2.6 percent developed tumors (*2*), which, although much lower than the present incidence, is nevertheless very high for this variety of tumor in laboratory animals. The variability of these animals indicates the advisability of using laboratory-bred animals in carcinogenesis studies so that all stages of the experiment may be controlled by the investigator. Recent demonstrations (*6*, *9*) that a microgram or less of 7,12-dimethylbenz[*a*]anthracene (9,10-dimethyl-1,2-benzanthracene) constitutes adequate initiation in croton-oil studies point to the extreme precautions that must be observed.

Initiation of carcinogenesis followed by croton oil has been demonstrated with a variety of stimuli, including fluorenylacetamide (acetylaminofluorene), urethan, β-irradiation, and thermal burning (*4*, *10–12*). Other sources of initiation, as yet undetected and unsuspected, may be involved in these studies and those of Roe (*7*). The findings clearly constitute an area for much thought and study.

Summary

1) Eight of 25 untreated albino mice obtained from a dealer developed multiple papillomas, sebaceous adenomas, and carcinomas.

2) The F_2 generation of these mice, bred in this laboratory, did not develop spontaneous tumors or show any response to repeated applications of croton oil.

3) A previous demonstration that croton oil exhibits carcinogenic action in these mice is discussed.

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Hemorrhagic Disease and Unusual Hepatic Lesions Associated with Intracerebral Passage of Rous Sarcoma Virus in Chicks [1,2]

VINCENT GROUPÉ,[3] FRANK J. RAUSCHER,[3] *and* W. RAY BRYAN,[4] *Institute of Microbiology, Rutgers University, The State University, New Brunswick, New Jersey, and National Cancer Institute,[5] Bethesda, Maryland*

Previous studies in this laboratory and elsewhere have shown that Rous sarcoma virus (RSV) propagated serially in the brain of newly hatched chicks (*1*, *2*) and killed them in relation to dose (*2*). Recent studies (*3*) have shown that the growth curve of RSV in chick brain was similar to that of other animal viruses and that the greatest virus content was found in chicks that were moribund or prostrate. Further, as with tumor tissue (*4*), the amount of virus extractable from the brain (*3*) was related to the infecting dose of RSV. Thus, chicks that were found moribund or prostrate after inoculation with less than 1 LD50 of virus yielded extracts of brain tissue that contained no extractable virus at all in 4 of 5 instances.

The experiments reported here describe the events that occurred during 30 serial intracerebral passages of RSV in newly hatched chicks. There was essentially no change in the potency of brain-tissue extracts during the first 18 passages but a progressive increase in the potency of brain tissue was observed after the 19th passage. Typical hemorrhagic disease, similar to that described by Duran-Reynals (*5*), was found in a few chicks on the 22nd passage and was observed with increasing frequency during subsequent passages. On the 25th passage of RSV, an unusual hepatic lesion was observed in addition to hemorrhagic disease, and, like hemorrhagic disease, continued to increase in frequency on subsequent passage. Such livers were small, vivid yellow in color, friable in texture, and contained large amounts of RSV. Additional quantitative studies were carried out showing that both hemorrhagic disease and hepatic lesions followed inoculation of an increased quantity of RSV, irrespective of the source of the virus.

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[3] Institute of Microbiology, Rutgers University.

[4] Member of the staff of the National Cancer Institute and Honorary Professor of Virology and Oncology at the Rutgers Institute of Microbiology.

[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

Materials and Methods

Rous sarcoma virus (RSV), also referred to as chicken tumor I agent, was obtained from the National Cancer Institute, where it has been maintained by serial passage in chickens. Unless otherwise indicated, unsexed White Leghorn chicks, 4 to 6 days of age, known to be free from *Salmonella pullorum* were used. Frozen, stable, standard virus, prepared by differential centrifugation from tumor tissue (*6*), was used to initiate the serial intracerebral passages and was stored in a dry-ice chest at −70° C.

Inoculation of chicks.—Chicks were placed under light ether anesthesia and inoculated intracerebrally into the right hemisphere with 0.05 ml. of inoculum. All chicks were observed daily for a period of 4 weeks, and each chick was examined at the time of death for the presence of the pathognomonic hemorrhagic lesion (*2*) in the meninges at the site of inoculation. Subcutaneous inoculation into the wing web was carried out as follows: Two-tenths ml. amounts were injected subcutaneously into the wing web. The needle was inserted through the muscle into the subcutaneous tissue, thus avoiding leakage. Only the left wing web was inoculated and the birds were examined daily for a period of 3 weeks. Typical tumors appeared 4 or more days after inoculation depending upon the potency of the inoculum.

Storage of tissue and preparation of extracts for serial passage.—Brain tissue was removed only from living or moribund chicks. Dead birds were discarded. Each brain to be used for subsequent passage was divided in half, and each half was placed in a separate ampoule that was sealed in an oxygen-gas flame. This procedure was carried out as follows: First, the brain was removed and the cerebrum was separated from the cerebellum; next, the cerebrum was cut in half transversely so that a portion of both hemispheres was included in each half; finally, the cerebellum was cut in half longitudinally. In this manner 2 identical pools of brain tissue were made available for assay. All tissue was stored in a dry-ice chest at −70° C. On the day of inoculation, half of each of 3 brains was thawed, pooled, and ground in a mortar with sand, and sufficient saline (containing 2% by volume of inactivated horse serum) was added to make a final concentration of 10 percent brain tissue by weight. This suspension was then clarified by centrifugation at 3000 r.p.m. for 20 minutes. The total time between removal of the ampoule from the dry-ice chest to the inoculation of the last chick did not exceed 2 hours. Beginning with the 5th passage, all inoculums were subjected to additional centrifugation to insure the removal of any intact cells that might have escaped disintegration by freezing, thawing, and grinding. This procedure (*4*) consisted of 3 additional cycles of centrifugation carried out as follows: The extract was placed in a Spinco Model L ultracentrifuge and centrifuged at 10,000 r.p.m. for 10 seconds. The middle third of the supernatant fluid was carefully removed after each centrifugation by means of a syringe equipped with a long hypodermic needle.

Bioassays.—The technical procedures used for determining the relative potencies of unknown extracts of tissue have recently been described in

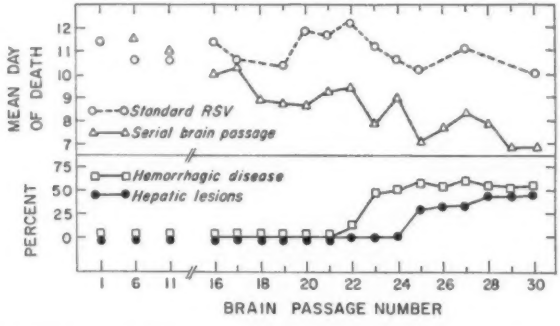
detail for both latent period of tumor production following subcutaneous inoculation (*7–10*) and time to death following intracerebral inoculation (*2, 3*). The reciprocal of time in days multiplied by 100 (*i.e.*, 100/days) has been found to be a suitable transformation for analysis of both time to death following intracerebral inoculation of virus and latent period for tumor production in the wing following subcutaneous inoculation of virus. Dose-response data were analyzed by the graphic methods recently illustrated in detail for tumor latent periods (*7*) and time-to-death responses (*2, 3*). The potencies of all tissue preparations were expressed in terms of percent relative to the observed potency of the standard, which was taken to be 100 percent in each assay. In this manner differences due to the marked fluctuation in the level of sensitivity of various test lots of chicks were eliminated by reference to a standard virus preparation within each test (*11*).

Results

Text-figure 1 summarizes the series of events that occurred during 30 serial passages of RSV in the brain of newly hatched chicks. These passages were initiated with standard virus lot CT581 (*6*), which was prepared from chicken tumor tissue by differential centrifugation. In the subsequent passages, groups of 15 to 30 chicks each were inoculated intracerebrally with a 10 percent suspension of brain tissue prepared from a pool of 3 brains collected from the preceding passage. Brains were removed only from chicks that were moribund or prostrate and showed typical pathognomonic hemorrhagic lesions at the site of inoculation. An effort was made to select for passage those chicks that succumbed early to the infection. Such brains were stored in sealed glass ampoules at −70° C., and brain tissue from each of 3 chicks was pooled, homogenized, and centrifuged prior to inoculation (*see* Materials and Methods). Beginning with the 21st serial brain passage, 3 extracts of individual brains were prepared and each was inoculated into 15 to 30 chicks. Brain tissue for subsequent passage was collected from the group of chicks that succumbed earliest and showed the highest incidence of both hemorrhagic disease and hepatic lesions. It was hoped that a further increase in potency could be obtained by this procedure. Complete necropsies were performed on all chicks used for subsequent passage, and during the last 10 passages necropsies were performed on all chicks. A parallel test on standard RSV, diluted 1:10, was included as part of the experimental procedure associated with each serial intracerebral passage of virus, and 15 to 30 chicks were inoculated intracerebrally with virus of standard lot CT581. The data summarized in text-figure 1 show that: (*a*) As anticipated from previous experience (*6*), host response to standard RSV varied appreciably among the various lots of test chicks. (*b*) For the first 17 intracerebral passages of RSV, there was no substantial deviation in potency from that of the standard virus. (*c*) Beginning with the 18th passage, there was a progressive increase in potency of brain-passage virus as compared with standard RSV. (*d*) On the 22nd passage, 13 percent of the chicks showed typi-

cal hemorrhagic disease that increased in incidence on subsequent passage to 66 percent by the 30th passage. (*e*) Hepatic lesions were observed in 30 percent of chicks inoculated with brain tissue collected from the 25th passage and continued to increase to 42 percent by the 30th passage. When hepatic lesions were present, they were invariably accompanied by hemorrhagic disease in the same organs and were characterized grossly by the vivid yellow color, reduced size, and friable texture of the livers. Typical hemorrhagic disease is shown pictorially in figure 1, and normal and diseased livers are shown in figures 2 and 3, respectively. Histologically, necrosis was not observed, but extensive irregular areas of obviously altered cells of an undetermined nature[6] were present (*see* figs. 4 and 5).

Table 1 shows the effect of serial brain passage of RSV on the virus content of the liver. In this experiment, groups of 10 chicks each were inoculated with standard RSV or with virus from the 35th brain passage. Both subcutaneous and intracerebral routes of inoculation were employed, as indicated in the table. Brain, liver, and tumor tissues were removed from each of 5 chicks in the various groups and the tissues were pooled and stored in the dry-ice chest. Later that week, the various pools were thawed and ground in a mortar with sand, and 10 percent suspensions by weight were prepared as previously described. Each of these suspensions was assayed for potency by intracerebral inoculation of chicks, the log percent potency being estimated by graphic procedures. The data show that: (*a*) There was little or no virus present in the liver when standard RSV was inoculated either subcutaneously or intracerebrally. (*b*) Virus was present in the brain following intracerebral but not subcutaneous inoculation of standard RSV. (*c*) A quantity of virus equal to that in tumor tissue itself was present in the livers of chicks inoculated intracerebrally with an extract from the 35th brain passage, and such livers showed hemorrhagic disease and hepatic lesions. Therefore, it is clear that the presence of hemorrhagic disease and hepatic lesions was associated with the presence of large quantities of virus in the liver.



TEXT-FIGURE 1.—Summary of 30 serial passages of RSV in chick brain.

[6] A detailed histologic study of these lesions has not been made. There are indications that they are neoplastic responses of the reticuloendothelial cells of the liver sinusoids to the circulating virus, but this interpretation is subject to revision after further investigation, which is obviously desirable.

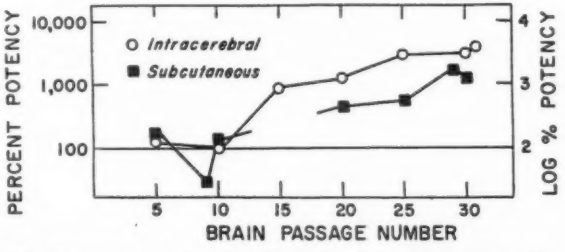
TABLE 1.—*Effect of serial brain passage of RSV on virus content of liver**

Inoculum	Route	Potency of:		
		Tumor (*log %*)	Brain (*log %*)	Liver (*log %*)
Standard RSV	SC	>3.5†	Neg.	0.3
Standard RSV	IC	—	>3.5	Neg.
35th Brain passage	IC	—	>3.5	>3.5‡

*SC=subcutaneous, IC=intracerebral, and Neg.=negative.
† Percent potency relative to standard RSV, lot CT632 (6), which was taken as 2.0 log percent or 100 percent.
‡ Livers showed hemmorrhagic disease and hepatic lesions.

In addition to the experiments just described, the tumor-producing potencies of the 5th, 9th, 10th, 20th, 25th, 29th, and 30th brain passages were determined by subcutaneous inoculation into the wing web (*see* Materials and Methods). Text-figure 2 shows the potencies of the various brain-tissue extracts relative to that of standard RSV. The data show that: (*a*) There was little or no change in potency following subcutaneous or intracerebral inoculation of brain extracts for the first 10 passages. (*b*) Beginning with the 15th intracerebral passage, there was a gradual and progressive increase in potency, as measured by intracerebral inoculation. (*c*) Similarly, there was a gradual increase in potency as measured by subcutaneous inoculation of brain-tissue extracts. This was first detected at the 20th brain passage and continued until the 30th passage, at which time the potencies, as measured by these 2 methods, were approximately equal.

Table 2 summarizes data from 6 representative experiments designed to show the relationship between the quantity of RSV inoculated and the incidence of hemorrhagic disease and hepatic lesions. In these experiments, standard RSV, extracts from the 29th and 30th brain passages, RSV from the 33rd brain passage concentrated 10 times in the ultracentrifuge, and an extract from a tumor of known high potency (1.6 log higher in potency than standard RSV as assayed in chicken wing web) were inoculated intracerebrally or subcutaneously, as indicated, into 1- to 6-day-old chicks. In all instances, groups of 30 to 35 chicks each were employed for intracerebral inoculation and groups of 15 to 20 chicks each were used for subcutaneous inoculation. All chicks were



TEXT-FIGURE 2.—Increase in potency of RSV associated with propagation of the virus in chick brain as measured by subcutaneous and intracerebral inoculation.

observed for a period of 3 to 4 weeks, and each chick was necropsied at the time of death or when the experiment was terminated. The data show that: (*a*) Neither hemorrhagic disease nor hepatic necrosis was observed when standard RSV was inoculated either intracerebrally or subcutaneously into 6-day-old chicks. (*b*) However, when 1-day-old chicks were employed, standard RSV produced both hemorrhagic disease and hepatic lesions following intracerebral but not subcutaneous inoculation. (*c*) Extracts of brain tissue collected from the 29th serial brain passage produced both hemorrhagic disease and hepatic lesions in chicks that were 6 days of age, and similar extracts from the 30th brain passage were markedly more effective in producing these lesions when inoculated into younger (1-day-old) chicks. (*d*) Ten times concentrated RSV from the 33rd brain passage produced hemorrhagic disease and hepatic lesions when inoculated by the subcutaneous route but only hemorrhagic disease when inoculated intracerebrally, in this particular experiment. (*e*) Extracts of tumor tissue of known high potency also produced both hemorrhagic disease and hepatic lesions in chicks 4 days of age when inoculated by the intracerebral route.

TABLE 2.—*Effect of amount of infecting dose of virus, route of inoculation, and age of host on production of hemorrhagic disease and hepatic lesions*

Inoculum		Age of chick (*days*)	Route*	Latent period (*days*)	Time to death (*days*)	Hemorrhagic disease (*percent*)	Hepatic lesions (*percent*)
Source	Dilution (*log*)						
Standard RSV†	−1	6	IC		9. 9	0	0
	−1	6	SC	4. 5		0	0
Standard RSV	−1	1	IC		10. 3	28	32
	−2	1	IC		12. 0	14	5
	−3	1	IC		13. 3	0	0
	−1	1	SC	4. 0		0	0
29th Brain passage RSV	−1	6	IC		6. 9	50	13
	−2	6	IC		10. 1	13	0
	−3	6	IC		11. 4	0	0
30th Brain passage RSV	−1	1	IC		7. 4	87	96
	−2	1	IC		9. 9	91	57
	−3	1	IC		10. 6	63	19
	−4	1	IC		12. 6	14	0
33rd Brain passage RSV 10× concentrated‡	0	4	IC		7. 4	71	0
	0	4	SC	3. 7		100	15
High-potency tumor tissue§	−1	4	IC		9. 3	47	27
	−2	4	IC		10. 9	8	0
	−3	4	IC		12. 7	0	0

*IC = intracerebral, SC = subcutaneous.

† Standard RSV, lot CT581 (7), was included as a part of all experiments with comparable results.

‡ Brain-tissue extract was centrifuged at 30,000 r.p.m. for 30 minutes in Spinco Model L ultracentrifuge and pellet was resuspended in 1:10 original volume.

§ The tumor-producing potency of this tumor extract was 1.6 log higher than standard, as assayed in chickens (*8*).

Discussion

The data presented in this report reveal the following facts: First, as anticipated (*5*), younger chicks (*i.e.*, 1 day of age) were the most susceptible to hemorrhagic disease and hepatic lesions. Second, hemorrhagic disease and hepatic lesions were produced more readily by intracerebral than by subcutaneous inoculation of RSV. Third, hemorrhagic disease and hepatic lesions were associated with the inoculation of large quantities of RSV. Fourth, more virus was required to produce hepatic lesions than was necessary to produce hemorrhagic disease. Fifth, the agent producing hemorrhagic disease and hepatic lesions was present in the original inoculum [lot CT581 of standard RSV (*6*)], in tumor tissue of known high potency, and in brain tissue from the 25th to 35th serial brain passage. It may be concluded that hemorrhagic disease and hepatic lesions are produced by the inoculation of large amounts of RSV, irrespective of its origin, and that hemorrhagic disease and hepatic lesions are associated with the presence of large quantities of virus in the liver.

Summary

The events that occurred during 35 serial intracerebral passages of Rous sarcoma virus (RSV) in newly hatched chicks are described. There was essentially no change in the potency of brain-tissue extracts during the first 18 passages. Beginning with the 19th passage there was a progressive increase in the potency of brain-tissue extracts. Typical hemorrhagic disease, similar to that described by Duran-Reynals, was observed in a few chicks on the 22nd passage and with increasing frequency during subsequent passages. On the 25th serial passage of RSV, hepatic lesions were observed in addition to hemorrhagic disease in the liver. Like hemorrhagic disease, it continued to increase with subsequent passages. Such livers were small, vivid yellow in color, and friable in texture. Hemorrhagic disease and hepatic lesions were associated with the presence of large quantities of RSV in the liver. Additional quantitative studies showed that both hemorrhagic disease and hepatic lesions followed inoculation of an increased quantity of RSV, irrespective of the origin of the virus.

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PLATE 5

FIGURE 1.—Hemorrhagic disease.

FIGURE 2.—Normal chicken liver.

FIGURE 3.—Hepatic lesions and hemorrhagic disease in chick liver.

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PLATE 6

FIGURE 4.—Liver lesion. *Note* sheets of pale-staining cells between which strands of liver parenchyma persist. Extensive congestion and some hemorrhage in adjacent liver tissue can be seen at *left*. Hematoxylin and eosin. × 105

FIGURE 5.—Higher magnification of liver lesion showing large straplike or ovoid cells with prominent vesicular nuclei and smaller darkly stained heterophil leukocytes. A fragment of uninvolved liver parenchyma is visible at *bottom right*. Hematoxylin and eosin. × 515

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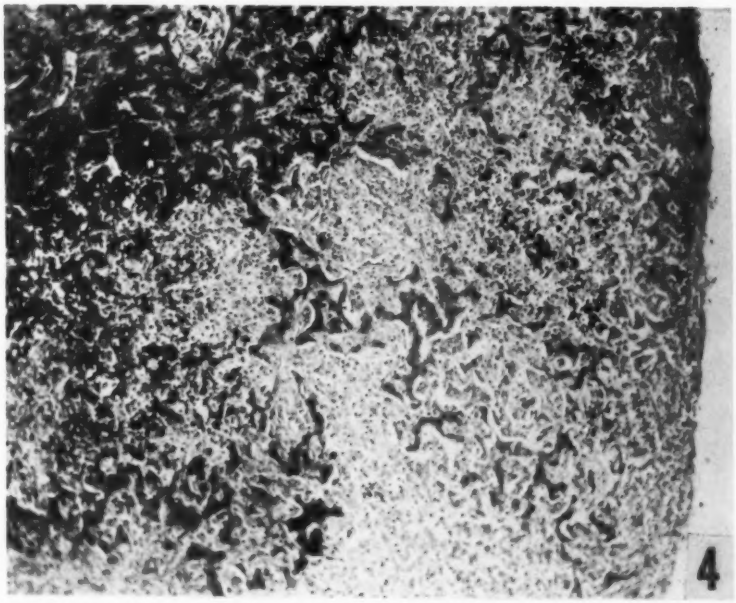
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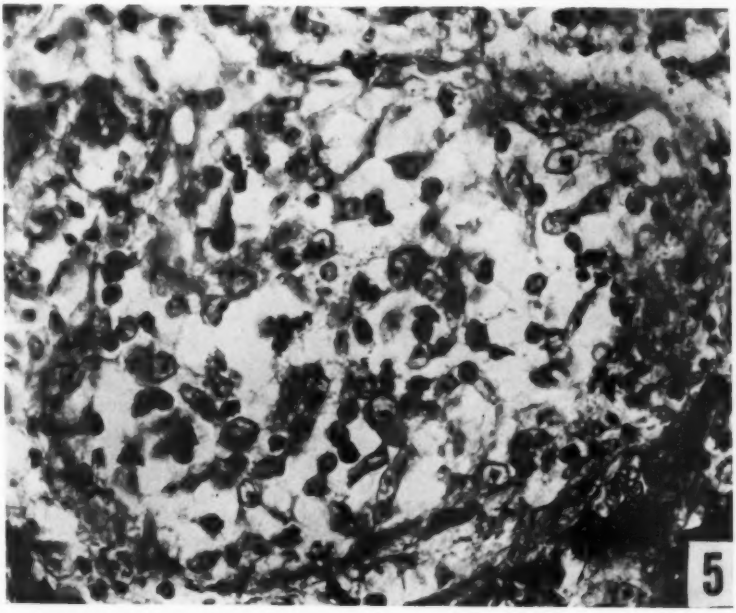
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Regression of HeLa Tumors in Cortisone-Treated Irradiated Rats following Infection with Coxsackie B3 Virus. A Histological and Cytochemical Study [1]

ROBERT LOVE, R. GERALD SUSKIND, *and* ROBERT J. HUEBNER,[2] *National Cancer Institute* [3] *and National Institute of Allergy and Infectious Diseases,*[3] *Bethesda, Maryland*

When suspensions of HeLa cells, prepared from tissue culture, were inoculated into the peritoneal cavity of cortisone-treated irradiated rats, large tumors developed (*1*). The tumors grew progressively for about 3 weeks but eventually regressed. Such tumors could be readily infected with various strains of Coxsackie B viruses without any striking effect on their growth. After several serial passages of the 4520 strain of Coxsackie B3 virus in rat HeLa tumors, growth of the virus was accompanied by a conspicuous oncolytic effect (*1*). Preliminary histological studies of virus-induced regression revealed that necrosis began at the periphery of tumor nodules and extended rapidly toward the center. The absence of this type of necrosis in uninfected tumors of the same age, even after inoculation of uninfected tissue cultures or cultures infected with other viruses (*1*), suggested that oncolysis might be the result of a specific cytopathogenic effect of the virus on the tumor cell.

In order to prove that virus infection is directly responsible for oncolysis, various other mechanisms must be excluded. Regression of virus-infected transplantable tumors may be caused, at least in part, by indirect stimulation of the local or systemic defenses of the host (*2–4*). Damage to tumor cells may also result from thrombosis and inflammation following the direct effects of virus infection on the normal cells of the host. The present experiments were designed to elucidate these points by a coordinated histological, cytological, and cytochemical analysis of growth and regression of HeLa tumors in the presence and absence of virus infection. The characterization of structural and chemical changes in HeLa cells infected with Coxsackie virus and under different adverse environmental conditions should be helpful in understanding the significance of cellular degeneration in carcinoma of the human cervix following infection with viruses (*5*).

Materials and Methods

General.—The type of rat, the irradiation procedure, and methods of administration of cortisone, inoculation of tumor and virus, and virus

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titration have been described elsewhere (*1*). In the present experiments, rats were inoculated with virus when they had developed palpable intraperitoneal tumors of more than 1 cm. in diameter. The virus inoculum (Coxsackie B3, R6MK3) was prepared from the third passage in monkey-kidney tissue culture of virus that had previously been through 6 successive passages in HeLa tumors in rats; it contained approximately 6 log tissue-culture infectious doses of virus per ml.

Rats were killed as indicated in the protocols. Tumors were removed and weighed; portions were taken for microscopic examination and for virus titration as previously described (*1*).

In the first experiment, to compare the growth and regression of uninfected and infected tumors, a total of 70 rats, bearing palpable tumors 10 days after intraperitoneal inoculation of HeLa cells, was divided into 2 groups. In 1 group, each of 35 rats was injected by the intraperitoneal route with 1 ml. of Coxsackie B3, R6MK3 virus. The remaining 35 rats were kept as controls.

The other experiments were carried out to confirm the reproducibility of virus-induced cell changes and their absence in uninfected controls and to make further cytochemical studies. Details of these experiments are given in table 1.

Technique for microscopic study.—Portions of tumor taken at random were minced finely with scissors, smeared on slides, and either air-dried or immersed in fixative while wet. Pieces of tumor were fixed and sections were prepared as described below.

Fragments of tumor were placed in 0.85 percent saline and in solutions of Janus green B and neutral red (*4*) and examined by direct and by phase microscopy. Smears fixed in 10 percent formalin saturated with mercuric chloride at 37° C. and paraffin sections from material fixed in formol sublimate were stained with hematoxylin and eosin and with Barrett's stain to show general cytological detail. Material was prepared for the demonstration of mitochondria by Kull's method (*6*) and of the Golgi apparatus by Kolatchew's method (*6*). After fixation in Bouin's fluid or formol sublimate, smears and paraffin sections were stained by Pappenheimer's procedure (*7*). Paraffin sections were also stained with van Gieson's stain for collagen and with resorcin fuchsin for elastic fibers.

The cytochemical properties of the tumors were investigated as indicated in table 2.

TABLE 1

Expt. no.	Virus inoculation			No. controls
	No. rats injected with virus	Dilution	Time of injection after tumor inoculation (*days*)	
2	5	Undiluted	11	1
3	5	10^{-1}	12	2
4	2	Undiluted	14	1

TABLE 2.—*Cytochemical procedures**

	Lipide		Acidic and neutral lipide	Phospholipide and unsaturated lipide	Cholesterol	Lipide aldehyde	Acetal phosphatide	Unsaturated fatty acids		1,2-Glycols
Fixation	Formol calcium	Formol sublimate	Formol calcium	Formol calcium	Formol calcium	Unfixed 10% formalin, formol sublimate	10% Formalin	10% Formalin	Formol sublimate	Formol sublimate
Method of preparation	Frozen section	Smear, paraffin section	Smear	Frozen section	Smear	Smear	Smear	Smear	Smear	Smear, paraffin frozen section
Staining method	Sudan black B, oil red O (*8*)	Sudan black B plus lipide extraction‡	Nile-blue sulfate (*9*)	Baker's acid hematin (*10*)	Schultz (*11*)	Schiff†	Plasmal	Schiff after 5 days in distilled water	Periodic acid-Schiff (*13*)	PAS
Remarks	The lipide nature of sudanophilic material in paraffin sections was confirmed by removal of stain with xylol and restaining with Sudan black		Compared with the results of Sudan-black staining	Control blocks fixed in Bouin and extracted in pyridine	Frozen sections of rat adrenal run as a positive control	Slides also blocked with phenylhydrazine before staining (*12*)	Preformed aldehydes blocked by phenylhydrazine	Preformed aldehydes and plasmals blocked		Staining prevented by acetylation

See footnotes at end of table.

TABLE 2.—*Cytochemical procedures**—Continued

	Periodic acid-Schiff (PAS) positive lipide	Unsaturated fatty acids	Glycogen	Mucopolysaccharides		Acid mucopolysaccharides and ribonucleic acid		Deoxyribonucleic acid	Sulfhydryl groups	Primary amino groups
Fixation	Formol sublimate	Formol sublimate	Formol sublimate	Formol sublimate	Formol sublimate	Formol sublimate		Formol sublimate	Formol sublimate	Formol sublimate
Method of preparation	Smear, paraffin section	Smear	Smear, paraffin section	Smear	Smear, paraffin section	Smear, paraffin section		Smear, paraffin section	Smear, paraffin section	Smear, paraffin section
Staining method	PAS after lipide extraction‡	Bromination(*13*) followed by PAS	Diastase, PAS, and lipide extraction‡, diastase, PAS	Lipide extraction‡, hyaluronidase, PAS	Mucicarmine	0.1% Toluidine blue	0.1% Ribonuclease 1 hr. at 37° C. followed by toluidine blue	Feulgen	Thioglycolate reduction and Bennett's RSR (*14*)	Ninhydrin-Schiff (*15*)
Remarks	Efficiency of lipide extraction checked by staining control with Sudan black			Compared with lipide extraction, PAS		Examined in water; then dehydrated in tertiary butyl alcohol. Control section for ribonuclease stored in distilled water 1 hr. at 37° C.			Control blocked for 2 hrs. in saturated aqueous mercuric chloride	Control blocked by acetylation (*15*)

*Except when indicated references for the methods employed have been given in a previous communication (*4*).
†All smears involving formol-sublimate fixation and the use of Schiff reagent fixed for 15 minutes only, to minimize effects of fixation.
‡Lipide was extracted by 12 hours' immersion in hot chloroform-methanol.

Observations

Gross Findings and Results of Virus Titration

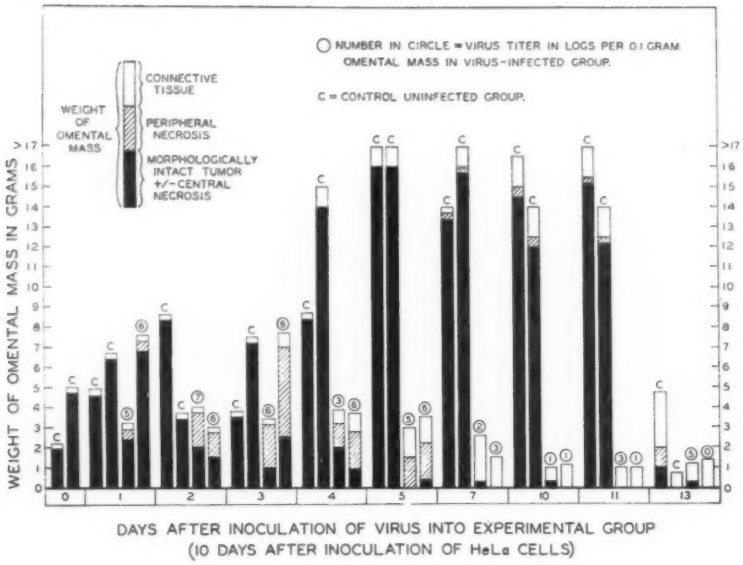
The macroscopic changes during the growth and regression of uninfected and virus-infected HeLa tumors in the rat have been described elsewhere (*1*). The tumors grew in the form of pearly-gray, solitary or conglomerate grapelike nodules in the omentum and mesentery and extended over the peritoneal surface of the abdominal viscera. Regression of virus-infected or uninfected tumors was characterized by the appearance of opaque white areas of necrosis followed by shrinkage and replacement by gray fibrous tissue. The manner in which regression was accelerated by virus infection is illustrated by the first experiment in text-figure 1. Comparison of the weights of infected and uninfected tumors from the 4th through the 11th day of infection revealed an increasing difference between the 2 groups. On the last day of observation, however, the weights of the control tumors dropped considerably and did not differ significantly from those of the infected group. Multiplication of virus in the tumor may be inferred from the high titers of virus that were demonstrated during the first 5 days after inoculation (text-fig. 1).

Histology

Experiment #1

Uninfected tumors.—Ten days after inoculation of HeLa cells into the rat, the tumors consisted of contiguous epithelial-like cells forming multiple circumscribed nodules, separated and surrounded by delicate strands of areolar tissue in which a very few macrophages were present (figs. 1 and 2). Occasional discrete necrotic tumor cells were observed in all tumors, and in most tumors small areas of necrosis were present in the center of some of the nodules (fig. 3). Minute zones of necrosis were first observed around the periphery of a few nodules, 17 days after implantation (text-fig. 1). After this, some degree of peripheral necrosis was almost invariably present (fig. 4), and the necrotic areas were invaded by macrophages and replaced by granulation tissue containing numerous fibroblasts and fine capillaries. Although macrophages were predominant in the inflammatory cellular reaction, neutrophil granulocytes and occasional giant cells were also observed (fig. 5). Lymphocytes and plasma cells were rarely found. In the older tumors the areas of central necrosis were larger and they frequently fused with the necrotic periphery, so that many nodules were completely necrotic. In 1 of the tumors, 23 days after implantation (text-fig. 1), the omental mass was composed entirely of connective tissue in which the fibers stained a faint pink by van Gieson's method.

Virus-infected tumors.—Necrosis in the center of the nodules and discrete necrotic cells were present as in the controls. One day after inoculation of virus, areas of necrosis were seen around the periphery of the tumor nodules (text-fig. 1); during the succeeding 4 days, necrosis progressed


NUMBER IN CIRCLE = VIRUS TITER IN LOGS PER 0.1 GRAM OMENTAL MASS IN VIRUS-INFECTED GROUP.
C = CONTROL UNINFECTED GROUP.
WEIGHT OF OMENTAL MASS
CONNECTIVE TISSUE
PERIPHERAL NECROSIS
MORPHOLOGICALLY INTACT TUMOR +/- CENTRAL NECROSIS
WEIGHT OF OMENTAL MASS IN GRAMS
DAYS AFTER INOCULATION OF VIRUS INTO EXPERIMENTAL GROUP
(10 DAYS AFTER INOCULATION OF HeLa CELLS)

TEXT-FIGURE 1.—Morphologic findings and virus titers during growth and regression of Coxsackie virus-infected and of uninfected HeLa tumors in the peritoneal cavity of cortisonized irradiated rats. The representation of relative amounts of connective tissue, necrosis, etc., in the tumors is an approximation based on the study of 3 sections of each tumor (expt. 1).

centripetally until the entire nodule was involved (fig. 6). The blood vessels were remarkably unaffected, and only minimal congestion and a few petechial hemorrhages were occasionally seen. There was virtually no inflammatory cellular reaction in the areas of necrosis during the first 3 days of virus infection (fig. 6). After this period, the inflammatory changes were similar to those described in the areas of peripheral necrosis in the older control tumors. Five of the 8 infected tumors, examined after the 5th day, were completely necrotic or entirely replaced by connective tissue (text-fig. 1). Although the omental mass that persisted in the remaining 3 rats was almost entirely fibrous, minute nodules of tumor remained (text-fig. 1).

Experiments #2, 3, and 4

The results of these experiments are summarzied in table 3. In experiment #2, minimal peripheral necrosis was noted in the control and in 1 of the 2 virus-infected tumors 2 days after inoculation of virus. Necrosis was more extensive in the other 11 infected tumors, especially on the 4th and 5th days of infection.

Cytology of the Rat HeLa Tumor Cell

The appearance of tumor cells in sections stained by Barrett's method and under the phase microscope is shown in figures 7 and 8. The nucleus

TABLE 3.—*Incidence of peripheral necrosis in uninfected and virus-infected rat HeLa tumors in experiments #2, 3, and 4*

Category	Days after inoculation of virus:				
	2	3	4	5	Total
Uninfected with peripheral necrosis	1	0	0	0	1
Uninfected without peripheral necrosis	2	1	0	0	3
Infected with peripheral necrosis	4	4	3	1	12
Infected without peripheral necrosis	0	0	0	0	0

was large and contained from 1 to 4 or more prominent nucleoli. Mitotic figures were very abundant throughout the period of observation, and bizarre forms and multipolar divisions were common. Occasional giant multinucleated tumor cells were seen. In preparations stained by Barrett's method, finely granular acidophilic parachromatin (or nuclear "sap") was demonstrable in the interphase nuclei of tumor cells. During prophase, aggregation of the parachromatin to form parachromatin bodies (*16*) was noted. At the end of prophase, the parachromatin bodies appeared to be liberated into the cytoplasm where they could be seen in metaphase, anaphase, and telophase (figs. 7 and 9). They were not found in the cytoplasm during interphase. The granules in the cytoplasm of dividing cells were also stained by toluidine blue. The resemblance of these granules to the amphophilic parachromatin bodies described in the cytoplasm of ascitic-tumor cells of mice suggests that they were of the same nature (*16*).

Many round or ovoid mitochondria were observed with the phase microscope (fig. 8) and, by direct microscopy, after staining with Janus green or by Kull's method (fig. 10). Small, elongated, translucent vesicles were visible under the phase microscope in the juxtanuclear zone of the cytoplasm (fig. 8). These were probably a negative image of the Golgi apparatus, because similar vesicles surrounded by sudanophilic and osmiophilic material were demonstrated in smears stained with Sudan black (fig. 11) and in Kolatchew preparations [5] (fig. 12). A few small granules in the juxtanuclear region of the cytoplasm were stained supravitally by neutral red.

In addition to staining the mitochondria and the Golgi lipide in smear preparations, Sudan black also colored a few larger globules in the cytoplasm of an occasional tumor cell. These globules were highly refractile in fresh preparations viewed with the phase microscope and were stained by oil red O in frozen sections.

Cytochemistry of the Rat HeLa Tumor Cell

The cytochemical properties of the nucleus did not differ from those of other tumor cells that have been analyzed (*4*, *16*), and, since the changes

[5] The material in the Golgi zone that was stained by Sudan black and by the Kolatchew's method will be referred to as the Golgi lipide.

observed in the nuclei of virus-infected cells were in no way unique, emphasis will be placed on the cytoplasmic organelles and inclusions (table 4).

The presence of acidic lipide in the mitochondria was demonstrated in smear preparations by their sudanophilia (fig. 13) and dark-blue coloration by the Nile-blue sulfate method of Cain (*9*). Although other substances in the sections reacted positively to Baker's acid hematin test for phospholipides, the mitochondria were unstained. Sulfhydryl or reduced disulfide groups were demonstrated in the mitochondria by Gomori's modification of Bennett's red sulfhydryl method (*14*). The mitochondria therefore contained protein-bound SH or SS groups and acidic lipide that may well be phospholipide despite the negative acid hematin test (*10*).

The Golgi lipide was not stained recognizably by Nile-blue sulfate but reacted positively to the PAS test in smear preparations (fig. 14). Preformed aldehydes and aldehydes secondary to the oxidation of unsaturated lipides could not be demonstrated in the Golgi lipide. The presence of acetal phosphatides was suggested by the following results: A positive plasmal reaction was obtained on material fixed in formalin, and the Golgi lipide was faintly stained by the Schiff reagent after fixation in formol sublimate. Since the direct Schiff test was negative on smears fixed in formalin, the latter reaction was presumably due to hydrolysis of acetal phosphatides during fixation. The performic acid-Schiff reaction for ethylenic groups was negative. Bromination reduced equally the intensity of staining of the Golgi lipide and that of all other PAS-positive material; therefore it did not provide any information about the presence of ethylenic linkages. The Golgi lipide was stained gray to blue-black by Baker's acid hematin method, which suggested the presence of phospholipide. No color or birefringence was detected in the Golgi or in any other lipide, and cholesterol could not be demonstrated. The positive PAS reaction of the Golgi lipide must be attributed to the presence of phospholipide, which, on the basis of the other tests, was probably a mixture of ester and acetal phosphatides. No sudanophilic material in the tumor cells survived extraction of smear preparations with hot chloroform-methanol. Minute granules of PAS-positive material persisted in the Golgi zone after extraction of lipide and diastase and hyaluronidase digestion. Similar granules were stained by the ninhydrin-Schiff procedure. Acetylation abolished the reaction with the ninhydrin- and periodic acid-Schiff techniques. The presence of primary amino groups, 1,2-glycols or, possibly, amino-alcohols in the Golgi region was demonstrated. In view of the increasing evidence that the histochemical PAS test is specific for 1,2-glycols (*17*), the reactive substance was probably glycoprotein.

The large cytoplasmic globules that were stained by fat-soluble dyes (table 4) were colored pink with Nile-blue sulfate and were, therefore, predominantly neutral lipide. They were also colored pale gray by Baker's acid hematin test, which is consistent with the presence of ethyl-

TABLE 4.—*Summary of cytochemical analysis of cytoplasmic particulates in uninfected and virus-infected HeLa cells grown in cortisone-treated irradiated rats**

Staining method	Mitochondria	Golgi lipide	Nonlipide Golgi component	Cytoplasmic lipide globules	Granules in cytoplasm during mitosis	Glycogen	Mucin	Basophilic granules in degenerating cells	Fuchsinophilic granules in paraffin sections
Sudan black B†	+	+	−	+	−	−	−	−	+
Nile-blue sulfate	Blue	−	−	Red	−	−	−	−	NT
Baker's acid hematin	−	Gray to black	−	Pale gray	−	−	−	−	NT
PAS	−	+	+	−	−	+	+	−	+
Lipide extraction, PAS	−	−	+	−	−	+	+	−	±
Lipide extraction, diastase, PAS	−	−	+	−	−	−	+	−	±
Plasmal	−	+	−	−	−	−	−	−	NT
Oxidation, Schiff‡	−	−	−	+	−	−	−	−	NT
Ninhydrin-Schiff	−	−	+	−	+	−	−	+	+
Bennett's RSR	+	−	−	−	+	−	−	−	+
Toluidine blue	−	−	−	−	+	−	−⊙	+	−
Ribonuclease, toluidine blue	−	−	−	−	−	−	−	−	−
Conclusion	Acidic lipide; protein-bound SS or SH groups	Ester and acetal phosphatide	Glycoprotein	Neutral unsaturated lipide	RNA protein	Glycogen	Neutral mucopolysaccharide	RNA protein	PAS-positive lipoprotein

*Abbreviations:
+=Stained, ±=poorly stained, −=not recognizably stained, NT=not tested, ⊙=not metachromatic.
†Smears fixed in formol sublimate.
‡Fixed in formalin; stored 5 days in water and treated with Schiff reagent.

enic groups. The neutral lipide was not stained by the PAS method, the direct Schiff test for preformed aldehydes, or the plasmal reaction for acetal phosphatides. After fixation in formalin and storage for 5 days in water, a few neutral lipide globules were Schiff-positive. When the slides were blocked with phenylhydrazine immediately after fixation and then stored for 5 days in water, the Schiff test was negative. This would suggest that some complex had been formed between formaldehyde and unsaturated lipide during fixation (*18*) and that oxidation of this compound in water gave rise to demonstrable aldehyde groups.

The amphophilic bodies present in the cytoplasm of cells in metaphase, anaphase, and telophase contained demonstrable primary amino groups and sulfur-containing amino acids; they were also stained by toluidine blue but not after ribonuclease digestion (table 4). Like the cytoplasmic parachromatin bodies of ascites tumors (*16*), they contained ribonucleoprotein.

The cytoplasm of a few cells contained numerous minute PAS-positive granules that could be removed by diastase digestion and therefore consisted of glycogen (table 4). Occasionally, coarse granules of mucinous material in the cytoplasm could be stained by mucicarmine (fig. 15). These granules were PAS-positive even after lipide extraction, diastase, and hyaluronidase digestion; they were not metachromatic and did not stain by the ninhydrin-Schiff or Bennett methods (table 4). Since no glycogen, lipide, protein, or acid mucopolysaccharides could be demonstrated in them, the "mucin" granules probably contained neutral mucopolysaccharide.

Degeneration of Rat HeLa Tumor Cells

Degenerative changes in the tumor cells may be divided into 3 general classes: *a*) Nonspecific degeneration, equally common in infected and uninfected tumors and encountered more frequently as the tumors grew older. This was present in all tumors with central or peripheral necrosis. *b*) Specific degeneration, present only in infected tumors showing peripheral necrosis on the 1st through the 5th day of infection and characterized by an increase in the Golgi lipide and eccentric displacement of the nucleus. *c*) Degeneration of doubtful significance, common in virus-infected tumors but also found in the absence of virus infection and characterized by the presence of basophilic and fuchsinophilic granules in the cytoplasm.

Class a—Nonspecific Degeneration

Changes leading to destruction of the nucleus were of 2 types. The more common form was characterized by a series of changes that began with regular shrinkage and loss of internal structure associated with loss of cytoplasmic basophilia (fig. 3); nuclear disintegration was completed by karyolysis or, less commonly, by karyorrhexis. This type of degeneration affected cells in interphase and in mitosis. The less frequent type

began with a primary change in the nucleus of cells in interphase without alteration of the cytoplasm until actual disruption of the nucleus had occurred. The process began with margination of the chromatin and parachromatin-body formation (fig. 16) similar to that described in ascites tumors (*19*). Shrinkage, peripheral displacement, and, finally, disintegration of the nucleoli was followed by karyorrhexis, loss of cytoplasmic basophilia, and fragmentation of the cell.

Occasional unusually large globular mitochondria were observed in cells with intact nuclei; they became increasingly common on prolonged observation of cells under the phase microscope. In cells showing nuclear degeneration, the mitochondria appeared to have disintegrated to form fine, dustlike granules (figs. 13 and 17); in the more advanced stages of degeneration, they could no longer be distinguished by phase microscopy (fig. 17) and could not be stained supravitally with Janus green or, after fixation, by Sudan black (fig. 18) or Kull's method.

Changes in the Golgi apparatus of uninfected tumor cells could not be followed readily by phase microscopy. Granules could not be stained supravitally with neutral red in cells with pyknotic nuclei. In smears stained by Sudan black or by the PAS method, and in sections stained with Baker's acid hematin or by the Kolatchew method, the Golgi apparatus of degenerating cells first became indistinct and later lost all affinity for stain (figs. 12, 14, and 18). Very faint uniform, diffuse sudanophilia and PAS-positive staining persisted in the cytoplasm of a few cells with pyknotic nuclei. After lipide extraction, the cytoplasm did not stain with Sudan black, but the minute nonlipide component of the Golgi apparatus was still detectable in occasional degenerating cells.

Around the periphery of areas of necrosis, many cells showed an increase in cytoplasmic neutral lipide globules that were stained gray by Baker's acid hematin method and black by the Kolatchew stain (fig. 12). In the center of the necrotic zones, practically no cytoplasmic lipide could be demonstrated in frozen sections stained by Sudan black or oil red O.

Glycogen and mucin did not appear to change significantly in amount or distribution during degeneration of the cell and were found extracellularly in areas of complete cytolysis.

Class b—Specific Degeneration

Regression of virus-infected tumors was invariably associated with the appearance of increasing numbers of cells showing a striking hypertrophy of the Golgi lipide (compare figs. 19 and 21 with 20 and 11). The increase in Golgi lipide could be demonstrated in 5 different ways. Under the phase microscope a circumscribed accumulation of finely granular material was observed in the Golgi zone of cells with eccentric pyknotic nuclei (figs. 22 and 23). The sudanophilic and osmiophilic nature of this material was demonstrated in smears stained with Sudan black (fig. 24) and in Kolatchew preparations (figs. 19 and 21). The minute lipide granules in the Golgi zone were also stained by the PAS method (fig. 25) and by Baker's acid hematin (fig. 26). The vacuolar portion of the Golgi appa-

ratus also appeared to be enlarged (fig. 23), but there was no definite change in the glycoprotein of the Golgi zone and supravital staining with neutral red was unaffected. The accumulation of Golgi lipide was at first clearly demarcated. It left a clear, structureless peripheral rim of basophilic cytoplasm and apparently displaced, or even indented, the shrunken pyknotic nucleus (figs. 21 and 23). Other nuclear changes accompanying the Golgi abnormality were in no way specific and culminated in karyolysis and fragmentation into dustlike particles. The mitochondria were included in the accumulation of Golgi lipide (fig. 22), where they underwent disintegration as in the uninfected cells. Cytoplasmic neutral lipide globules were uncommon in cells showing the Golgi change.

Class c—Degeneration of Doubtful Significance

When smear preparations were stained with toluidine blue or Barrett's stain, cells with altered Golgi lipide were recognized by the presence of a weakly staining juxtanuclear zone of cytoplasm in which basophilic or slightly metachromatic granules were sometimes seen (figs. 27 and 28). Basophilic granules were invariably present in some cells when the Golgi change was detected, but the proportion of cells containing granules was extremely variable and could not be correlated with the amount of virus demonstrable in the tumor. Similar granules could not be demonstrated in the juxtanuclear zone in smear preparations of uninfected cells, but a very prolonged search did reveal a few basophilic granules in the cytoplasm of a degenerating cell. The cytochemical properties of the basophilic granules are summarized in table 4. The basophilia was destroyed by ribonuclease digestion. The granules were stained faintly by the ninhydrin-Schiff method and presumably consisted of ribonucleoprotein. When smears were stained by Pappenheimer's method, fuchsinophilic granules were irregularly stained in intact and degenerating cells of uninfected and infected tumors.

At this point, the results of staining smears must be contrasted with those obtained with paraffin sections. In paraffin sections of infected tumors, basophilic cytoplasmic granules could be demonstrated in some of the degenerating cells in areas of peripheral necrosis. Basophilic granules could not be seen in paraffin sections of the majority of uninfected tumors. In sections of 2 virus-infected and 1 uninfected tumor, basophilic cytoplasmic granules were present in small groups of necrotic cells in the center of some of the tumor nodules (fig. 29). These granules contained demonstrable ribonucleoprotein and were cytochemically indistinguishable from those present in areas of peripheral necrosis in the virus-infected tumors. When sections of tumors were stained by Pappenheimer's method, fuchsinophilic cytoplasmic material was observed in areas where the cells were known to contain basophilic granules. Fuchsinophilic granules were very minute in the areas of peripheral necrosis of infected tumors (fig. 30). In zones of central necrosis, the fuchsinophilic material was coarsely granular and often fused to form

large masses of material that occupied the whole cytoplasm (figs. 31 and 32). Closer study of sections stained with toluidine blue revealed that the basophilic granules were frequently hollow (fig. 29) and were not present in cells that were completely filled with fuchsinophilic material. Comparisons of sections stained by Sudan black (fig. 33), the periodic acid-Schiff, ninhydrin-Schiff, and red sulfhydryl methods with those stained by Pappenheimer's technique revealed that the "Pappenheimer" granules consisted of PAS-positive lipoprotein (table 4). The appearance of hollow basophilic particles would seem to be produced by a coating of cytoplasmic material around and between the lipoprotein granules, which, as they increased in number and size, finally fused and occupied the entire cytoplasm.

Discussion

A certain amount of necrosis around the periphery of tumor nodules was a feature of all regressing rat HeLa tumors. In the absence of virus infection, peripheral necrosis was rarely found until 2 to 3 weeks after implantation of HeLa cells. Degeneration of the neoplastic cells was always the primary event, and this was followed by infiltration by macrophages, phagocytosis of cell debris, and fibrosis. The absence of lymphocytes and plasma cells makes it unlikely that oncolysis was the result of a local immune response. Although ischemia was probably a factor in the production of central necrosis, it could hardly be the cause of necrosis around the periphery, which was nearest to the blood vessels of the host. Therefore, in the uninfected tumors, peripheral necrosis was more probably brought about by the action of a circulating cytopathic substance, such as a toxin or an antibody.

If virus-induced oncolysis was merely an acceleration of the spontaneous process of regression, cytological and histological changes like those in the uninfected tumors would be expected. The cytological changes were, however, different in the virus-infected tumors. Peripheral necrosis was more extensive and more rapidly progressive and was invariably present 1 to 5 days after inoculation of virus. The initial peripheral distribution of necrosis suggests the operation of a blood-borne agent. Subsequent studies have confirmed that rat HeLa tumors are infected by the blood stream, since the same pattern of necrosis developed when virus was introduced by the intravenous, intracerebral, or oral route (*20*). None of the cytological changes described in susceptible animals infected with Coxsackie viruses (*7*, *21*, *22*) were found in the stroma of the tumors or in fragments of pancreas in sections of the present material. When normal rats were inoculated with Coxsackie B3 virus, there was no evidence of multiplication of virus in the omentum and pancreas (*1*). Neither damage to blood vessels nor thrombosis was detected, and cytolysis of tumor cells was the first change observed.

The nature of the changes in the virus-infected cells provides further evidence that oncolysis was caused by the direct cytopathic effect of the virus. While the presence of degenerating cells in which the Golgi lipide is increased is not diagnostic of Coxsackie B3 virus infection,

there is considerable evidence that an increase in Golgi lipide may be produced by intracellular proliferation of virus. The Golgi abnormality was not found in uninfected tumors and was present only during the period when maximal amounts of virus were demonstrable in the tumors. Hypertrophy of the Golgi apparatus of virus-infected cells has been reported in canine distemper (*23*), myxomatosis (*24*), Rift Valley fever (*25*), vaccinia (*26*), fowl pox (*27*), and louping ill (*28*). Recently, Reissig *et al.* (*29*) have reported the development of a discrete granular mass in the cytoplasm of monkey kidney cells in tissue culture infected with poliomyelitis virus. Although cytochemical studies were not done, the phase-contrast pictures of infected cells strongly suggest that the Golgi lipide was increased (*29*). An increase in the Golgi lipide has also been found in lymphoma cells of the chicken (*4*) and in Ehrlich ascites-tumor cells (*30*) after infection with a number of different viruses.

The results clearly demonstrate the limited value of Pappenheimer's stain in the diagnosis of Coxsackie B virus infections. The evidence presented here indicates that certain types of lipide may stain with fuchsin and that "Pappenheimer" granules can be readily demonstrated in uninfected material (figs. 31 and 32).

The basophilic granules in HeLa cells may be of the same nature as those described by Levaditi in nerve cells infected with Coxsackie B virus (*31*). The formation of basophilic granules in occasional uninfected HeLa cells was attributed to fragmentation of the cytoplasm during the accumulation of lipide deposits. A similar mechanism may be responsible for the basophilic granularity in the enlarged Golgi zone of virus-infected cells, but the possibility of the formation of virus inclusions cannot be excluded. Although estimations of the size of Coxsackie B viruses (*32*) indicate that they are less than 40 mμ in diameter, aggregations of virus could be visible under the light microscope. The presence of RNA in the basophilic granules is also consistent with the presence of virus in them.

Summary

1) The histological features of tumors produced by inoculation of HeLa cells into cortisone-treated irradiated rats are described.

2) Spontaneous regression of HeLa tumors in the rat began 2 to 3 weeks after implantation and was brought about by a combination of increasing central and peripheral necrosis, followed by phagocytosis and replacement fibrosis.

3) Some cytochemical properties of the mitochondria, Golgi apparatus, cytoplasmic lipide globules, glycogen, and mucin granules of intact and degenerating tumor cells are analyzed. Granules of ribonucleoprotein, believed to be derived from the nuclear parachromatin, were found in the cytoplasm of rat HeLa tumor cells in metaphase, anaphase, and telophase.

4) Rapid oncolysis was produced by infection of growing tumors with a strain of Coxsackie B3 virus that had been adapted to the rat HeLa

tumor. Virus-induced regression was characterized by the development of extensive areas of peripheral necrosis in which the degenerating cells showed a striking increase in the Golgi lipide, sometimes associated with the presence of granules of ribonucleoprotein in the juxtanuclear region of the cytoplasm. The nature of the RNA protein granules is discussed, and it is concluded that their presence is not absolutely diagnostic of Coxsackie B3 virus infection.

5) Fuchsinophilic granules, similar to those described by Pappenheimer in mice infected with a Coxsackie B virus, were demonstrated in infected tumor cells but were also found in uninfected cells.

6) The results suggest that oncolysis following infection with Coxsackie B3 virus was directly attributable to the cytopathic action of virus on the tumor cell.

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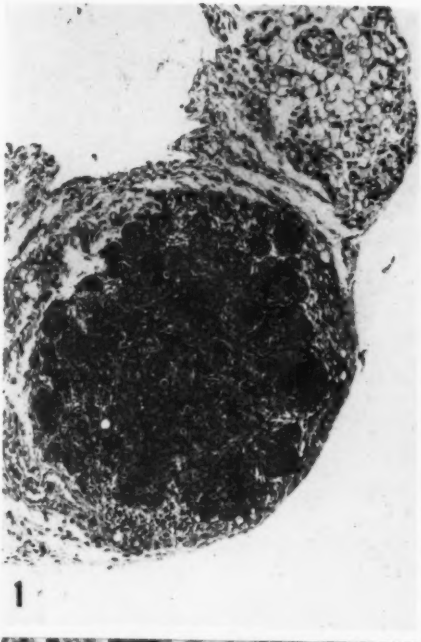
PLATE 7

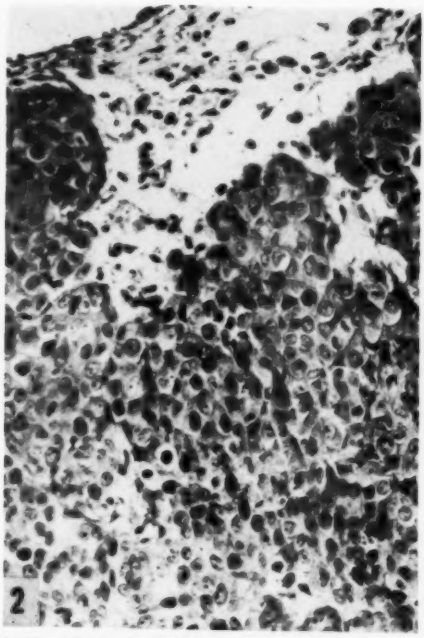
FIGURE 1.—Tumor in the omentum of a rat, 10 days after inoculation of HeLa cells. No fibrosis or inflammatory cellular reaction. Hematoxylin and eosin. × 108

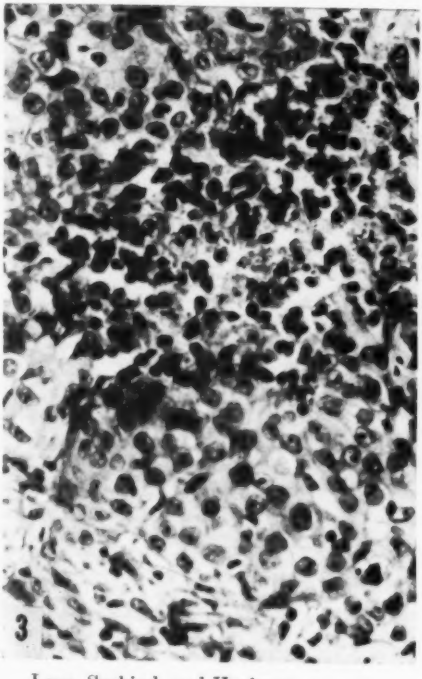
FIGURE 2.—Tumor in omentum of a rat, 12 days after inoculation of HeLa cells. Numerous mitotic figures are present. No necrosis. There are a few macrophages in the areolar tissue around the periphery. Barrett's stain. × 210

FIGURE 3.—HeLa tumor in a rat, 19 days after inoculation of HeLa cells. Necrosis in the center of a nodule. The cells at the periphery (*bottom*) are morphologically intact. Barrett's stain. × 290

FIGURE 4.—Necrosis at the periphery (*left*) of a tumor in the rat, 19 days after inoculation of HeLa cells. Hematoxylin and eosin. × 290







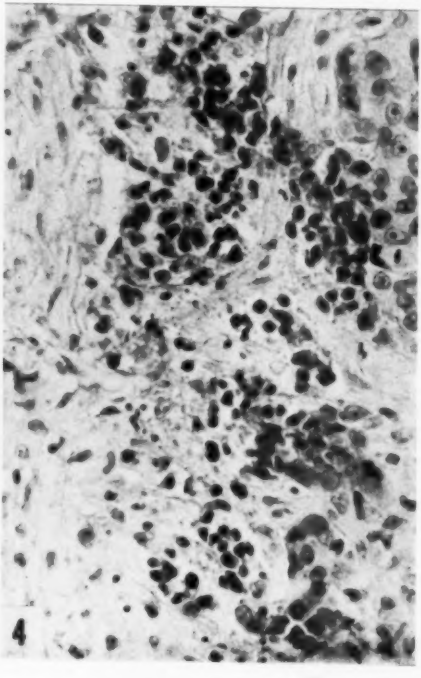


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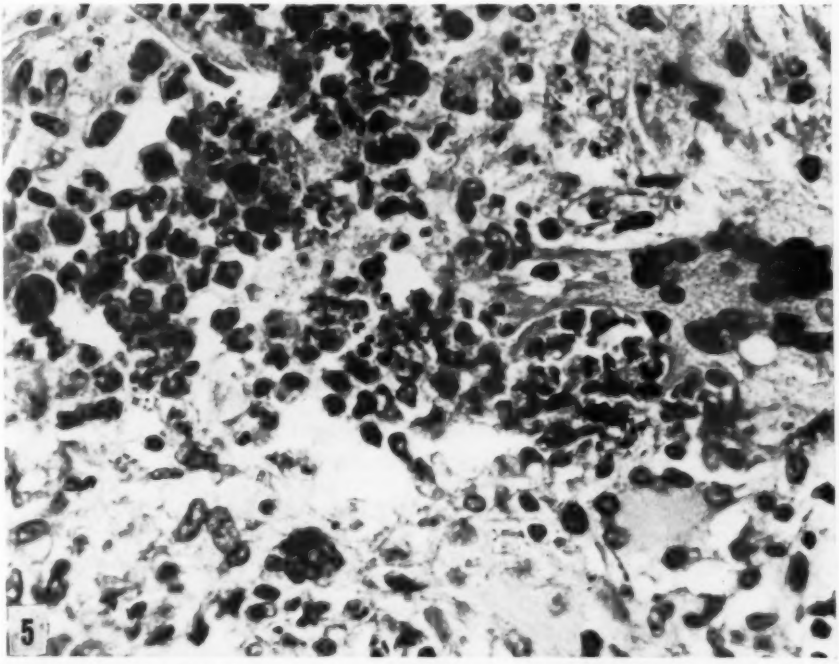
PLATE 8

FIGURE 5.—Invasion of necrotic tumor cells by macrophages, neutrophil granulocytes, and giant cells, 23 days after inoculation of HeLa cells. Hematoxylin and eosin. × 610

FIGURE 6.—Extensive necrosis in a 12-day-old rat HeLa tumor, 2 days after intraperitoneal inoculation of Coxsackie B3 virus. *Note* that a few intact tumor cells remain in the center of some of the nodules and that disintegration of tumor cells is more advanced at the periphery of each nodule. No inflammatory cellular reaction. Compare this tumor with an uninfected tumor of the same age (fig. 2). Barrett's stain. × 210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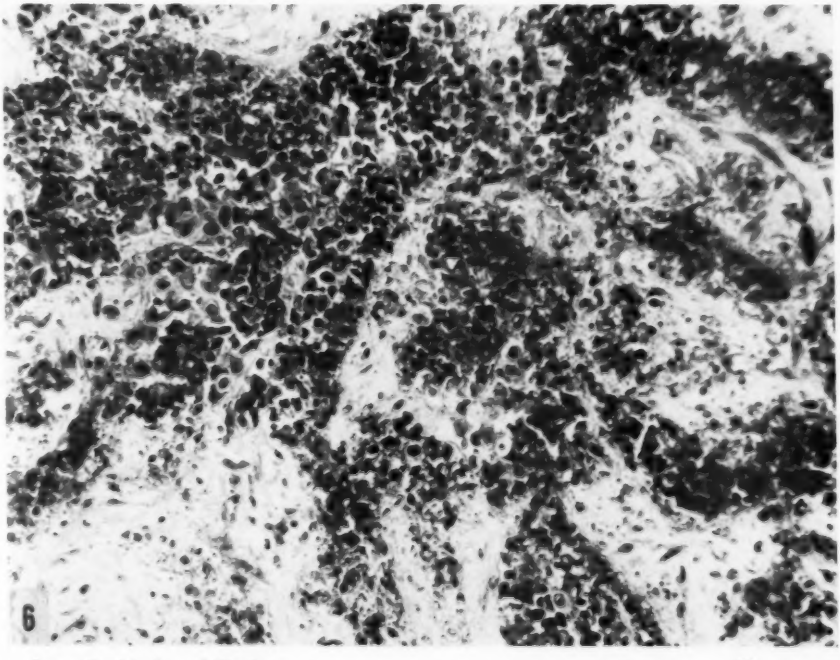
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PLATE 9

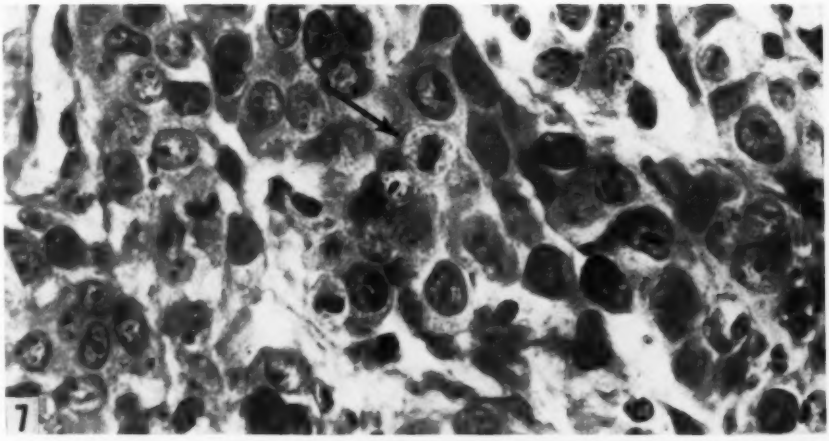
FIGURE 7.—Higher magnification of a 12-day-old uninfected HeLa tumor. *Note* granules in the cytoplasm of cells in mitosis. These are most clearly visible in the cell in metaphase indicated by *arrow*. Barrett's stain. × 610

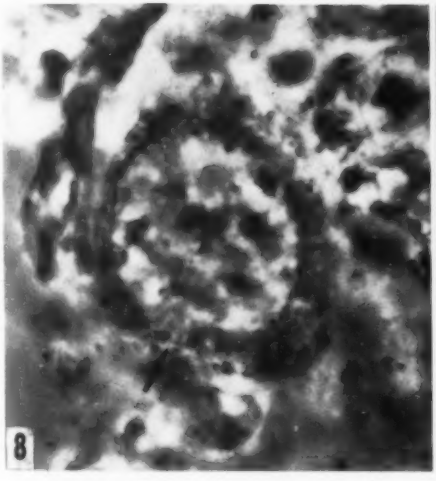
FIGURE 8.—Phase-contrast photograph of an uninfected HeLa tumor cell from the rat. *Note* round or ovoid mitochondria in the cytoplasm and a small translucent vesicle (*arrow*) close to the nuclear membrane. × 1600

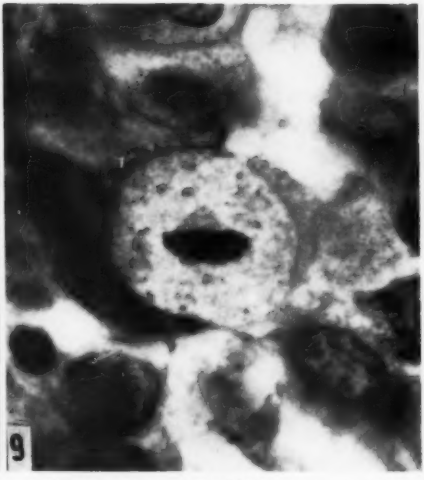
FIGURE 9.—Rat HeLa tumor. *Note* granules in the cytoplasm of a cell in mitosis. No granules are visible in the cytoplasm of cells in interphase. Barrett's stain. × 1600

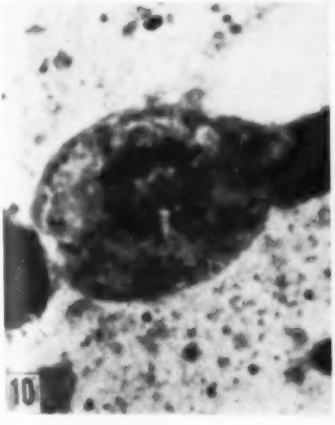
FIGURE 10.—Mitochondria in a rat HeLa cell in metaphase. Kull preparation. × 1700

FIGURE 11.—Smear preparation of rat HeLa cells stained with Sudan black and focused to show the Golgi lipide. × 1700









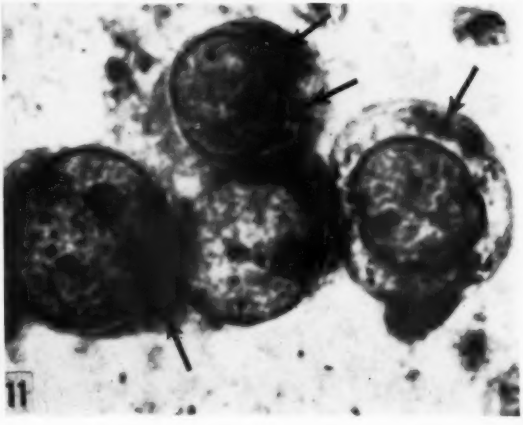


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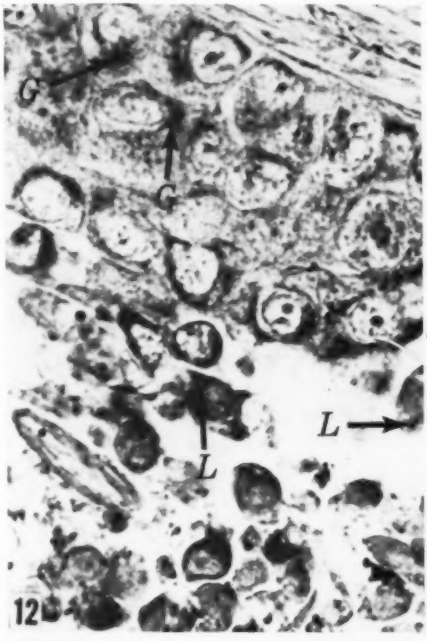
PLATE 10

FIGURE 12.—Eighteen-day-old uninfected rat HeLa tumor showing osmiophilic material in intact and degenerating cells. The Golgi lipide (G) and occasional large lipide globules (L) are stained. Kolatchew preparation. × 735

FIGURE 13.—Smear preparation of uninfected rat HeLa cells stained with Sudan black to show mitochondria. The mitochondria cannot be distinguished in 2 cells with pyknotic nuclei (*lower right*) in which larger lipide granules are present. × 1700

FIGURE 14.—Smear preparation of uninfected rat HeLa cells stained by the periodic acid-Schiff method. *Note* PAS-positive vesicular structures (*arrows*) in intact cells. The cytoplasm of 2 cells with pyknotic nuclei is PAS-negative. Counterstained with hematoxylin. × 2080

FIGURE 15.—Group of rat HeLa cells containing coarse granules of mucin. Mucicarmine. × 735



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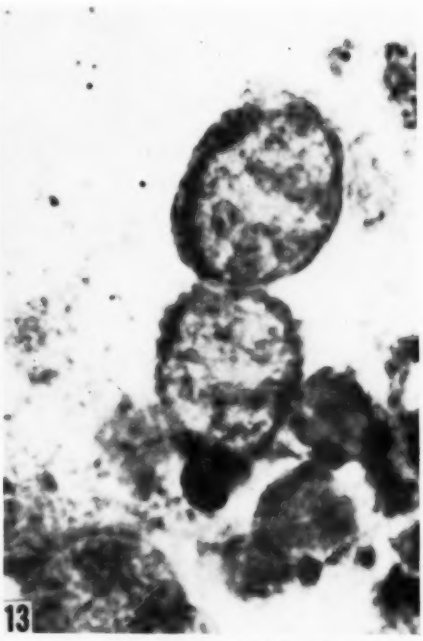

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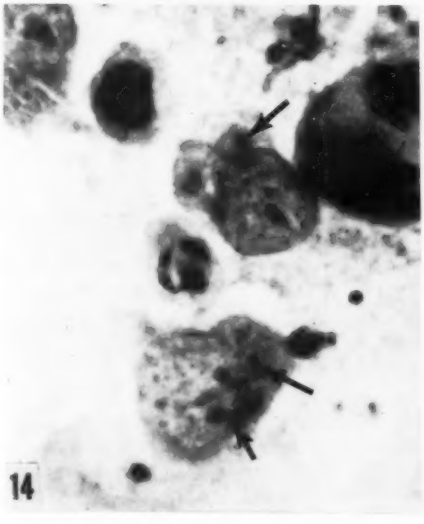

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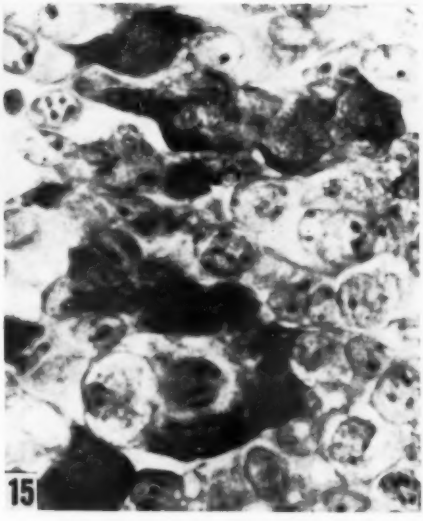

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PLATE 11

FIGURE 16.—Margination of chromatin with peripheral displacement of nucleolus (N) and prominent parachromatin body (P). Uninfected rat HeLa tumor. Barrett's stain. × 1600

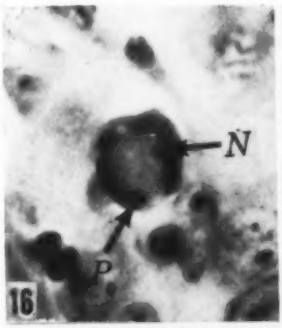
FIGURE 17.—Phase-contrast photograph of 2 degenerating cells from an uninfected rat HeLa tumor. *Note* fine, dustlike granules in the cytoplasm of the *upper* cell. The mitochondria cannot be distinguished in the cytoplasm of the *lower* cell. × 1600

FIGURE 18.—Smear preparation stained with Sudan black showing 2 degenerating cells from an uninfected rat HeLa tumor. The mitochondria and the Golgi lipide cannot be distinguished. Counterstained with carmalum. × 1700

FIGURE 19.—Low-power view of a Kolatchew preparation of an 18-day-old tumor infected 4 days previously with Coxsackie B3 virus. *Note* striking increase in osmiophilic material in areas of peripheral necrosis. × 210

FIGURE 20.—Uninfected tumor of the same age as that shown in figure 19. There is very little osmiophilic material in the intact and degenerating cells. Kolatchew preparation. × 210

FIGURE 21.—Higher magnification of the tumor shown in figure 20. *Note* accumulation of osmiophilic material in the cytoplasm and displacement, and even indentation, of the nucleus. Compare with the uninfected tumor shown in figure 12. Kolatchew preparation. × 735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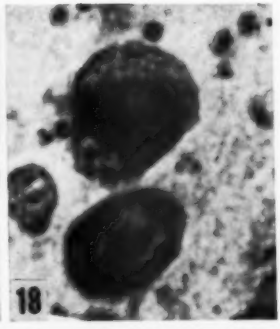
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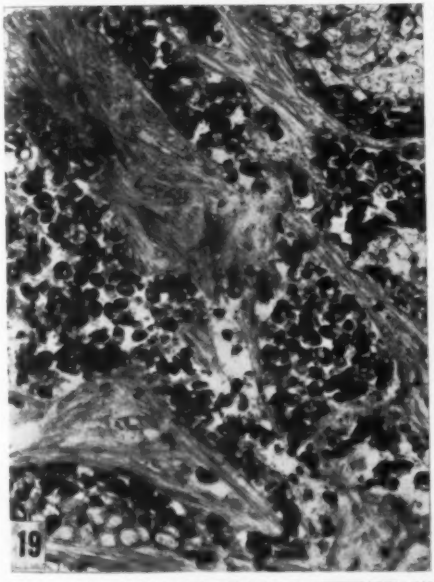
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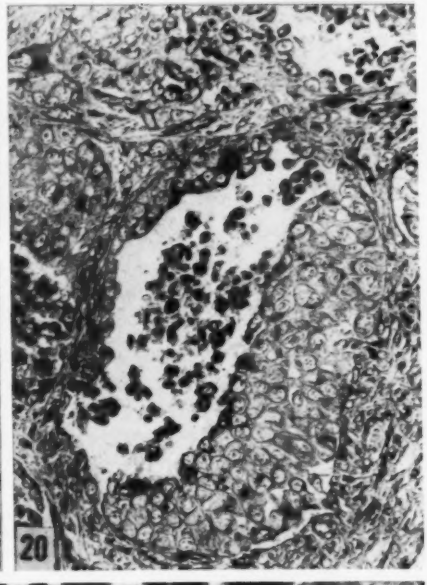
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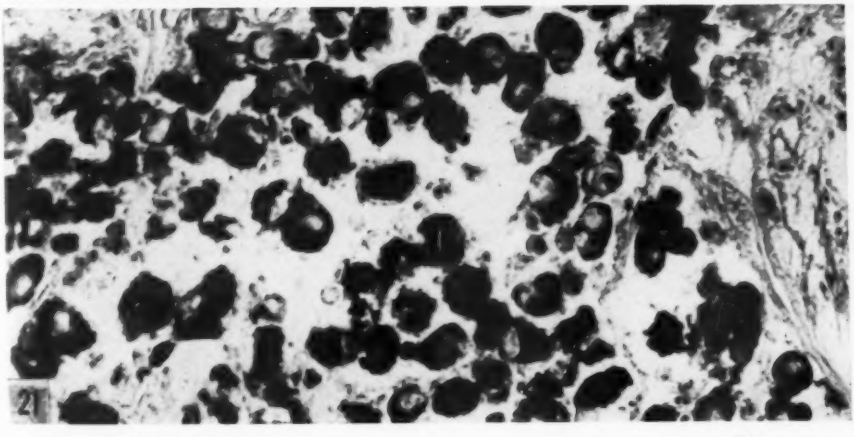
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PLATE 12

FIGURE 22.—Phase-contrast photograph of a rat HeLa tumor cell, 3 days after infection with virus. *Note* circumscribed accumulation of granular material in the upper half of the cytoplasm, in which the mitochondria appear to be included. The lower part of the cytoplasm appears to be structureless. × 1600

FIGURE 23.—Rat HeLa tumor cell, 4 days after virus infection. Displacement and indentation of pyknotic nucleus (N) by finely granular vacuolated material in the cytoplasm. The mitochondria can no longer be recognized. Phase contrast. × 1600

FIGURE 24.—Smear of a virus-infected rat HeLa tumor stained with Sudan black and carmalum. The pyknotic nucleus (N) is eccentrically displaced and there is a circumscribed accumulation of sudanophilic material in the cytoplasm. × 2080

FIGURE 25.—Smear of the tumor shown in figure 24 stained by the PAS method and counterstained with hematoxylin. *Note* resemblance of the PAS-positive material in the cytoplasm to the lipide shown in figure 24. × 2080

FIGURE 26.—Section of a virus-infected tumor stained by Baker's acid hematin method. *Note* black-staining lipide in the cytoplasm. The nucleus (N) is unstained. × 1600

FIGURE 27.—Smear of the infected tumor shown in figures 24 and 25 stained by Barrett's method. Minute basophilic granules are present in the perinuclear area of the cytoplasm. Compare with figures 22 through 26. × 2080

FIGURE 28.—Smear stained with toluidine blue. Minute basophilic granules in the cytoplasm of a degenerating virus-infected tumor cell. × 1600

FIGURE 29.—Paraffin section of an uninfected tumor stained with toluidine blue. Large basophilic granules in the cytoplasm. At least 1 of the granules (H) appears to be hollow. × 1700

FIGURE 30.—Paraffin section showing very fine fuchsinophilic cytoplasmic granules in an area of necrosis at the periphery of a virus-infected tumor. Pappenheimer's stain. × 1600

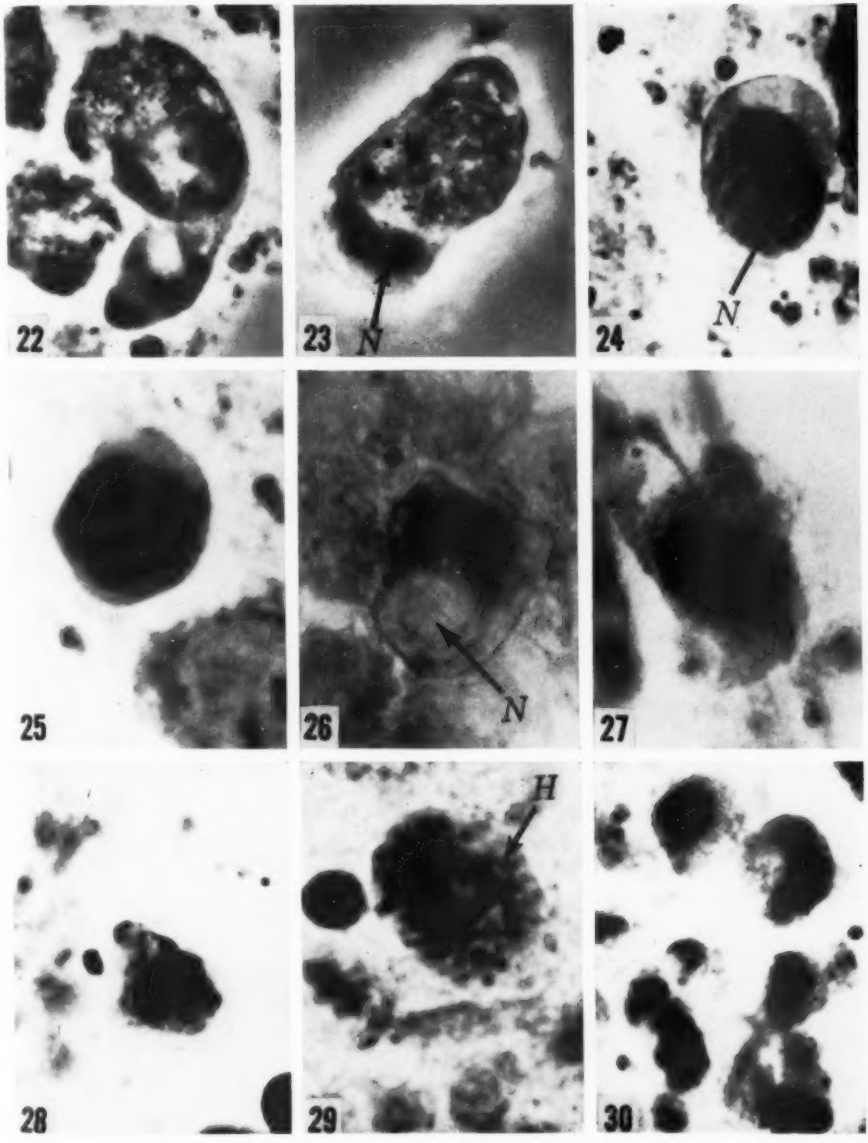
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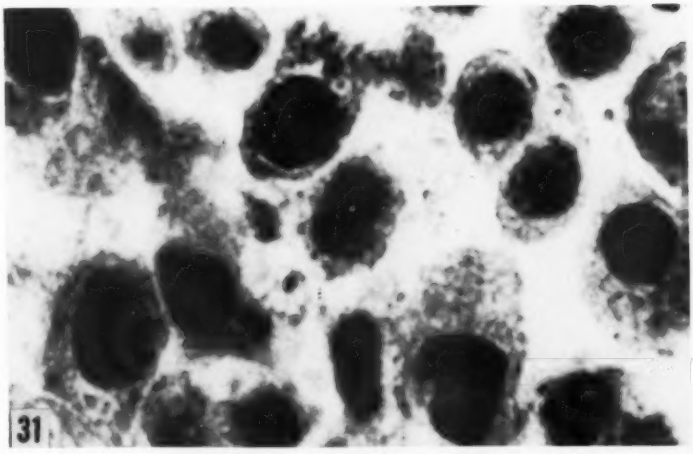
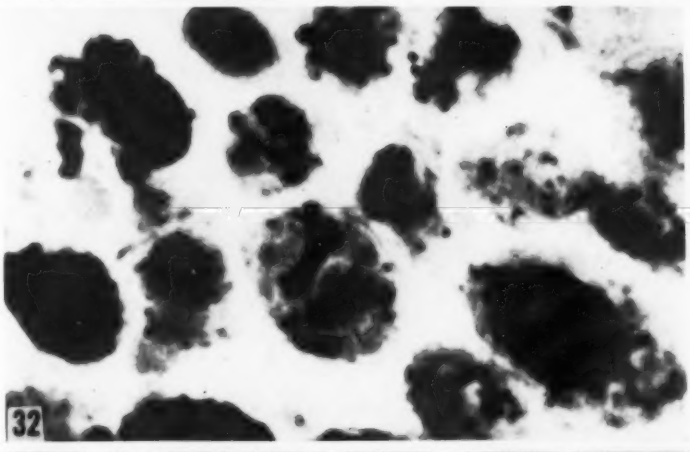
PLATE 13

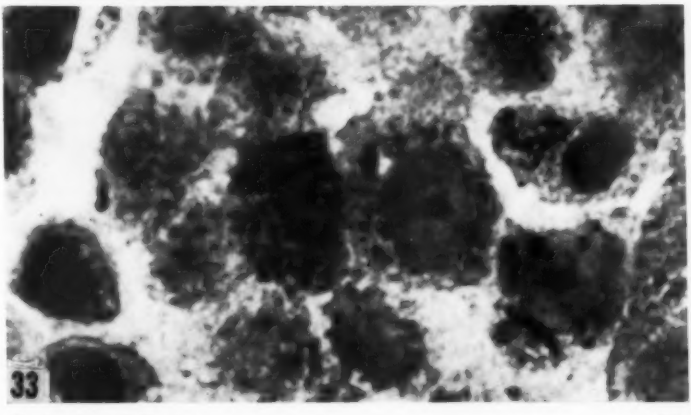
FIGURE 31.—Paraffin section showing fuchsinophilic granules in the cytoplasm of cells in an area of necrosis in the center of an uninfected tumor. Pappenheimer's stain. × 1700

FIGURE 32.—Another area of the section shown in figure 31. *Note* gradation in size of the fuchsinophilic granules, some of which fill the entire cytoplasm. Pappenheimer's stain. × 1700

FIGURE 33.—Paraffin section stained with Sudan black and carmalum. Sudanophilic granules in the cytoplasm of cells in the area of necrosis depicted in figures 31 and 32. × 1700







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Appearance and Persistence of Fluorescent Material in Tumor Tissue after Tetracycline Administration [1, 2]

DAVID P. RALL, TI LI LOO, MONTAGUE LANE, *and* MARGARET G. KELLY, *Clinical Pharmacology and Experimental Therapeutics Section, National Cancer Institute,*[3] *Bethesda, Maryland*

Extensive pharmacologic studies of a riboflavine antagonist, sodium-6,7-dimethyl-9-ethyl-isoalloxazine succinate (U 6538), which fluoresces yellow under ultraviolet (UV) light, were conducted on a patient with metastatic breast carcinoma. When this patient came to autopsy and tissue specimens were examined in UV light, portions of the tumor fluoresced yellow, while normal organs exhibited only the usual autofluorescence. Since a study of riboflavine antagonists closely related to U 6538 had been conducted in this laboratory (*1*), autopsy material was available from other patients who had received these agents. Tumor tissue from these patients as well as tissue from patients who had not received the riboflavine analogues was examined in UV light for yellow fluorescence. A few of the tumors from patients who had been given a riboflavine analogue exhibited yellow fluorescence under UV light. However, similar fluorescence was observed also in tumors of patients who had not received these compounds. U 6538 was then administered to mice carrying implants of sarcoma 37. Most tissues, including the tumor, showed fluorescence under UV light for the first 4 to 8 hours after the drug. By 12 hours, however, all induced fluorescence had disappeared from the tumor as well as the other tissues. Therefore, the medications received by the patients whose tumors fluoresced were reviewed, and this suggested that tetracycline, which also shows yellow fluorescence in solution, might be the cause of the yellow fluorescence in the tumors.

Tetracycline then was given to mice with implants of sarcoma 37. The tumor tissue and, in addition, the bones and teeth of these animals exhibited a bright yellow fluorescence under UV light that persisted in these areas until the death of the tumor-bearing animals 2 to 3 weeks later. Except for the bones and teeth (*2*), none of the other normal tissues showed persistence of the characteristic yellow fluorescence under UV light. Relatively little information is available concerning the distribution in

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[2] The authors wish to thank Dr. Harold Petering of the Upjohn Company, Kalamazoo, Mich., for the sodium-6,7-dimethyl-9-ethyl-isoalloxazine succinate; Dr. Richard Swarm of the Laboratory of Pathology, National Cancer Institute, for his generous assistance in surveying the autopsy specimens; and Dr. Clyde O. Brindley, Jr., of the General Medicine Branch, National Cancer Institute, for his assistance.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

the body of antibiotics of the tetracycline series despite wide clinical usage. The tissue distribution of these compounds has been studied by UV fluorescence microscopy, with the use of yellow fluorescence of the tetracyclines as an indication of their presence in tissues (*3–5*). Studies have also been reported in which microbiological assay has been employed to ascertain the distribution of these compounds (*6*, *7*). These reports have concluded that the tetracyclines are widely distributed in the body, have no special affinity for any tissue, and tend to accumulate in the organs through which they are excreted, such as the intestine, liver, and kidney.

Materials and Methods

In the preliminary experiment, the riboflavine analogue or tetracycline was given intraperitoneally to 22 gm. CA F_1 mice bearing intramuscular implants of sarcoma 37 (*8*). The drugs were given daily for 2 to 5 days, and the animals were sacrificed at intervals thereafter. Most of the other experiments were also performed on CA F_1 mice with sarcoma 37; some information pertaining to other rodent tumors is given in table 4. Chlortetracycline and oxytetracycline, as well as tetracycline,[4] were dissolved in 0.9 percent saline, generally 10 mg. per ml., and used within 1 hour. Intraperitoneal injections of the drugs were given daily for 2 to 5 days beginning on the 6th day of tumor growth. For slow-growing tumors, this schedule was repeated at weekly intervals. Animals were sacrificed at intervals at least 48 hours after the last injection of drug. Complete autopsies were performed, and gross specimens of all organs, including normal and neoplastic tissues, were examined under ultraviolet light.[5] No attempt was made to quantify the intensity of the fluorescence seen.

Results

Solutions of tetracycline and of the riboflavine analogue fluoresced yellow under UV light. Inspection, under UV light, of mice that had received the riboflavine analogue showed yellow fluorescence in most tissues, including tumor, soon after drug administration; however, the fluorescence disappeared rapidly and completely within a few hours. Following tetracycline, yellow fluorescence appeared in the tumor and most other tissues and persisted in the tumor and in the bones and teeth. Further studies were therefore undertaken with tetracycline.

The tissue distribution of tetracycline following an intraperitoneal injection of 2 mg. to mice with 7-day-old implants of sarcoma 37 was studied by the presence of the induced yellow fluorescence. For the first 6 hours the drug appeared in most areas except the brain. Tumor tissue, the liver, parts of the intestines, and bony tissue all retained the material for about 24 hours. Thereafter, the yellow fluorescence was seen only in tumor and bone where it persisted, essentially undiminished, until the death of the

[4] Tetracycline (Achromycin), lots #7-6211 and #6-6380, and chlortetracycline (Aureomycin), lot #7-6212, were kindly supplied by Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y. Oxytetracycline (Terramycin), lot #WBW 517361, was kindly supplied by Chas. Pfizer & Co., Inc., Brooklyn, N. Y. In addition, random samples of clinical preparations were used and found to be effective.

[5] Model SC-5041, 125 watt, 3660 Å, Hanovia ultraviolet light, Hanovia Chemical & Mfg. Co., Newark, N. J.

animal in 10 to 20 days. The fluorescence in the tumors was distributed diffusely in the more peripheral areas and was not apparent in the grossly hemorrhagic or necrotic zones. The fluorescent areas covered about 50 percent of the cut surface of the tumor (fig. 1.)

One mg. of tetracycline per mouse produced this phenomenon in about half the tumors (table 1); twice this amount, in single or divided doses, was consistently effective. Oral or subcutaneous administration of tetracycline was also effective in causing the appearance and persistence of this fluorescent material. When tetracycline was given prior to the 5th day of tumor growth no fluorescence was seen in the implants. When given after the 6th day, however, it was uniformly effective in producing fluorescence (table 2). All 3 of the tetracycline antibiotics in wide usage today—tetracycline, chlortetracycline, and oxytetracycline—were qualitatively similar in causing the appearance and persistence of fluorescence in sarcoma 37 (table 3).

TABLE 1.—*Fluorescence in sarcoma 37 after tetracycline**

Total dose (*mg./mouse*)	No. daily injections	No. with fluorescence / No. injected
12	4	12/12
8	4	12/12
4	2–4	11/12
2	1–4	17/18
1	1–2	6/12
0. 5	1	2/6

*Tetracycline HCl given intraperitoneally to CA F_1 mice with 7-day-old sarcoma 37 implants. The mice were autopsied under UV light 3 days after injection.

TABLE 2.—*Effect of age of tumor on fluorescence after tetracycline**

Tumor age (*days*)	No. with fluorescence / No. injected
3	0/4
5	1/8
6	6/7
7	10/10
8	10/10

*Tetracycline HCl, 3 mg. per mouse, given intraperitoneally on day indicated. The mice were autopsied under UV light 3 days after drug administration.

Many of the other tumors investigated also showed persistence of this fluorescent material after tetracycline administration (table 4). Mice with the ascitic form of sarcoma 37, Crocker sarcoma 180, and the Krebs-2 carcinoma were given the tetracycline subcutaneously. Both the free cells, as determined by gross inspection of the packed material after centrifugation of the ascitic fluid, and the solid portions of the ascitic tumors showed bright yellow fluorescence. Fluorescent material appeared and persisted in 14 of 19 different experimental neoplasms in mice. All 7

TABLE 3.—*Fluorescence in sarcoma 37 after chlortetracycline and oxytetracycline**

Drug	Total dose (*mg./mouse*)	No. with fluorescence / No. injected
Chlortetracycline	12	12/12
	8	6/6
	2	3/5
Oxytetracycline	12	12/12
	8	5/5
	2	3/4

*Drugs given intraperitoneally 4 times daily to CA F_1 mice with 7-day-old sarcoma 37 implants. The mice were autopsied under UV light 72 hours after drug administration.

sarcomas and carcinomas of the mouse, including 3 newly arisen osteogenic sarcomas, showed fluorescence. Two hepatomas and 4 of 7 leukemias and lymphomas that were studied showed the yellow fluorescent material. In general, sarcomas and carcinomas were positive as were some lymphomas and leukemias. Melanomas did not show persistence of fluorescent material after tetracycline administration.

Administration of tetracycline had no apparent effect on the growth of the tumors, although the experiments were not designed to critically evaluate anti-tumor activity.

TABLE 4.—*Fluorescence in transplantable tumors after tetracycline**

Tumor†	Implant site‡	Animal host
Tumors showing fluorescence		
Sarcoma 37	IM, IP, or SC	CA F_1 mouse
Sarcoma 180	IM or IP	CDBA mouse
Krebs-2 carcinoma	IM or IP	CA F_1 mouse
Ehrlich carcinoma	IM	CDBA mouse
Barrett carcinoma	IM	C3H mouse
Osteogenic sarcomas (*3*)	IM	C3H, AKR mouse
Yoshida sarcoma	IM	Japanese rat
Hepatoma 129P	IM	C3H mouse
Hepatoma 134	IM	C3H mouse
Hepatoma 7974	IM	Japanese rat
Lymphoma #4	IM	CA F_1 mouse
Lymphoma #2	IM	CA F_1 mouse
Leukemia P-288	IM	CDBA mouse
Leukemia P-388	IM or IP	CDBA mouse
Tumors not showing fluorescence		
Lymphoma #1	IM	CA F_1 mouse
Leukemia 2416	IM	C57BL mouse
Leukemia 1210	SC or IP	CDBA mouse
Harding-Passey melanoma	IM	CA F_1 mouse
Cloudman melanoma S91	IM	CA F_1 mouse

*Tetracycline HCl, 2 mg. per mouse or 10 mg. per rat, given intraperitoneally or subcutaneously 2 or 3 times per week beginning when tumors were 7 days old. Repeated at weekly intervals for slow-growing tumors. Animals autopsied at least 2 days after last tetracycline injection.

†Sarcoma 37, Crocker sarcoma 180, the Barrett carcinoma, the Harding-Passey melanoma, Cloudman melanoma S91, and leukemia 1210 were kindly supplied by Dr. M. J. Shear; the Krebs-2 and Ehrlich carcinomas, Yoshida sarcoma, hepatomas, lymphomas, and leukemias P-288 and 2416 were kindly supplied by Dr. Morris Belkin. Dr. Thelma Dunn and Dr. A. Hilberg kindly gave us the osteogenic sarcomas.

‡IM = intramuscular; IP = intraperitoneal; SC = subcutaneous.

Preliminary observations on neoplastic tissue obtained at autopsy from patients who had received 1 of the tetracycline antibiotics also suggest that certain carcinomas showed persistence of the fluorescent material, whereas leukemias did not. Persistence of fluorescent material was observed at autopsy on 2 patients with carcinoma of the breast, whereas no fluorescence was seen in 2 patients with acute leukemia.

Discussion

The possibility of the selective or preferential localization of drugs within neoplastic tissue has been the subject of a number of investigations and continues to be a problem of considerable interest (*9–15*). Such drugs could serve as important tools in many phases of cancer research. The therapeutic potentialities inherent in a drug that is concentrated in tumor tissue are obvious. Biochemical or histologic investigations into the mechanism of the selective uptake of drugs by tumors could serve to illuminate some of the differences between normal and neoplastic tissue. Such agents also might aid in the diagnosis of cancer.

This report presents data demonstrating that the tetracyclines appear to localize in tumor tissue. Once these compounds are present in the neoplasm they persist for a long period of time. These agents are unique in the number and the variety of tumors in which they localize. Of particular interest is the specificity and duration of their persistence in tumor tissue.

At present, it is not known to what degree the tetracyclines are concentrated in tumor tissue, although the distribution and localization of fluorescent materials can be followed in the experimental animal with apparent simplicity under UV light. This technique has severe limitations in terms of estimating drug concentration in tissue. Many of the factors influencing the intensity of fluorescence, such as *p*H and the presence or absence of activators or quenchers, can vary significantly in biological tissue, and the possibility of metabolites of differing fluorescent properties precludes any attempt to quantify drug concentration in tissue by this simple method.

Too little information is available, at present, to permit even speculation as to the mechanism of persistence of fluorescent material in tumor tissue after tetracycline administration. However, research on the chemistry of the fluorescent material in tumor tissue is in progress in this laboratory, and studies are under way on the histology of the tumors in which the fluorescence persists and the areas in which it is located.

Summary

A material that shows bright yellow fluorescence under ultraviolet light appears in the tumor tissue of a number of experimental tumors in mice and rats, as well as in human tumors, after administration of tetracycline, chlortetracycline, or oxytetracycline. In the experimental tumors this fluorescent material appeared after doses of about 50 mg. per

kg. and persisted for at least 10 to 20 days, until the death of the animal. Similar material was noted to persist in bone but not in other tissues.

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Plate 14

Figure 1.—Photograph under 3600 Å light of 12-day-old intramuscular implants of sarcoma 180 in CA F_1 mice. Animal *on left* given no drug. Animal *on right* given 3 daily 50 mg. per kg. injections of tetracycline intraperitoneally, ending 3 days prior to the photograph.

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Bone Localization of the Tetracyclines [1]

ROBERT A. MILCH,[2] DAVID P. RALL,[3] *and* JOHN E. TOBIE,[4, 5] *National Cancer Institute*[6] *and National Institute of Allergy and Infectious Diseases*,[6] *Bethesda, Maryland*

In the preceding communication (*1*), data have been presented which suggest that fluorescence may be detected not only in certain tumors but also in some skeletal tissues following administration of tetracycline. The present report concerns the tissue distribution of fluorescence following administration of antibiotics of the tetracycline series to a variety of laboratory animals, with special reference to the skeletal localization of the fluorophor.[7]

Material and Methods

Exploratory studies were undertaken in mice, rats, guinea pigs, rabbits, and dogs. Animals of the several species were given a parenteral dose of the tetracyclines, varying from 5 to 200 mg. per kg., and were sacrificed at intervals thereafter.[8]

Sprague-Dawley rats, weighing 80 to 150 gm., and New Zealand white rabbits, weighing 1 to 3 kg., were employed in tissue-specificity and time-dose studies. Rats were given an intraperitoneal dose of 50 mg. per kg. of tetracycline and were sacrificed at intervals from 30 minutes to 10 weeks after a single injection. A similar dose was given intravenously to Sprague-Dawley rats and CA F_1 mice, and the animals were sacrificed at 30-second intervals after the start of the injection. Doses of tetracycline from 0.1 to 50 mg. per kg. were given intraperitoneally to Sprague-Dawley rats weighing 140 gm. in an attempt to ascertain the minimal

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[2] Surgery Branch, National Cancer Institute. *Present address:* Department of Surgery, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Mass.

[3] Clinical Pharmacology and Experimental Therapeutics Section, National Cancer Institute.

[4] Laboratory of Immunology, National Institute of Allergy and Infectious Diseases.

[5] The writers would like to express their sincere thanks to Dr. Leon Sokoloff, National Institute of Arthritis and Metabolic Diseases, for his kind suggestions and aid during the preparation of the manuscript.

[6] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[7] For simplicity, the fluorophor in bone is here called "tetracycline," though it should be appreciated that the fluorescent material may be a metabolic derivative of the administered compound. Studies on the chemical properties of the bone fluorophor are currently in progress.

[8] Tetracycline (Achromycin), lots #7-6211 and #6-6380, and chlortetracycline (Aureomycin), lot #7-6212, were supplied by Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y. Other lots were employed with similar results in studies other than those presented in this communication. Oxytetracycline (Terramycin), lot #WBW 517361, was supplied by Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

dose giving rise to grossly detectable bone fluorescence. A 4-hour time interval following injection of tetracycline was arbitrarily selected as the time for comparison and the lower end of the femur was arbitrarily selected as the bone to be examined.

Tissues were removed immediately after sacrifice and examined grossly under a 125-watt ultraviolet light source (3660 Å).[9] They were then stored in the fresh state at −8° C. for subsequent microscopic examination, after it had been determined that storage at this temperature did not appear to alter either the intensity or the localization of the induced fluorescence.

Fresh sections of undecalcified bone were obtained by freezing the bone specimens with freon in a model DT-31 Techni-Freeze refrigeration apparatus.[10] Sections of approximately 15 μ were obtained with a Bausch & Lomb clinical freezing microtome. Only occasional sections were satisfactory for microscopic examination.

The bone sections were mounted on glass slides in glycerin-gelatin (1:1), covered with glass coverslips, and examined in dark field with a Leitz Ortholux microscope equipped with a photographic attachment. The ultraviolet source was a 1000-watt AH-6 General Electric high-pressure mercury lamp and the 3650 Å line obtained with a 6 mm. UG-1 filter. The lens of a 35 mm. Leica camera was replaced by a Wratten 2B gelatin filter and photographs were obtained with Kodak Tri-X (TX-135) safety film. Exposure time varied between 5 seconds and 5 minutes; the most satisfactory photographs were obtained with an exposure of approximately 45 seconds.

Results

In control animals of all species, a faint bluish autofluorescence was apparent in most tissues and was especially marked in blood vessels, the cartilaginous extremities of long bones, the hyaline cartilages of the axial skeleton and trachea, and in the brain. Brilliant yellow-gold fluorescence was grossly apparent in the bones of all animal species, irrespective of the specific dosage schedule employed following tetracycline, chlortetracycline, and oxytetracycline administration. This induced fluorescence was easily and clearly distinguishable from normal autofluorescence.

Thirty minutes after intraperitoneal injection of tetracycline in the rat, a diffuse yellow-gold fluorescence was present in all animals throughout the peritoneal cavity and the tissues of the abdominal wall. Between 30 minutes and 6 hours, all soft tissues progressively lost the induced fluorescence. Fluorescence persisted, however, in both long and flat bones and in teeth, and 12 hours following tetracycline, grossly evident induced fluorescence appeared to be confined to bone and teeth in virtually all animals. The brain showed unaltered autofluorescence at all times throughout the experimental period. An occasional liver showed induced

[9] Model SC-5041 Hanovia ultraviolet light, Hanovia Chemical & Mfg. Co., Newark, N. J.
[10] Scientific Products Division, American Hospital Supply Co., Evanston, Ill.

fluorescence after 12 hours and an occasional femur showed yellow-gold fluorescence along the weight-bearing portions of the condyles and the femoral head at this time. These findings, however, were inconstant.

When once deposited in bone, the induced fluorescence did not appear to be displaced throughout the remainder of the 10-week experimental period. Grossly, young and adult bone could be differentiated readily on the basis of the intensity of the emitted light as well as its distribution and were readily distinguished from the control bones (fig. 1). Microscopically, the localization of the fluorescent material was quite specific in all animals. In ultraviolet light articular and epiphyseal cartilage presented a deep-blue or bluish-gray autofluorescence, which could be quite precisely differentiated from the brilliant yellow-gold fluorescence in regions of new bone proliferation (fig. 2). Periosteal and endosteal surfaces in these regions characteristically presented induced fluorescence, and this was more prominent in young than in adult animals.

In rats weighing 150 gm., unequivocal bone fluorescence could be detected within 30 seconds of the start of an intravenous injection of 50 mg. per kg. of tetracycline. Striking fluorescence was apparent in the metaphysis immediately subjacent to the epiphyseal plate of the femur 4 hours after intraperitoneal injection with doses of 5 mg. per kg. of body weight, and minimal fluorescence could be detected grossly with doses in the range of 0.3 mg. per kg.

Discussion

The present observations suggest not only that each of the 3 commonly available tetracyclines localize specifically in bone but also that bone fluorescence following their parenteral administration occurs at an extremely rapid rate, even when remarkably small amounts of the compounds are given. Furthermore, when once apparent in bone, fluorescence persists for relatively prolonged periods of time. A precise evaluation of the biological half-life of the fluorescent material, however, can be undertaken only when adequate analytical methods, now lacking, have been developed.

The general distribution of fluorescence along the periosteal and endosteal surfaces of bone suggests a direct relationship between localization in these regions and bone blood supply. Clearly, surface bone, being most recently deposited, is in closest anatomic proximity to tissue fluids and, thus, is more likely to be presented with relatively greater amounts of the administered material than more internally located, physiologically more inert bone crystals.

The precise mechanism whereby localization occurs remains unelucidated. It may be surmised, however, if only on the basis of the lack of persistence of fluorescent material in tissues richly supplied by body fluids, such as heart, liver, kidney, and spleen, that mere availability of the administered compound is not the sole mechanism involved in its persistence in osseous tissue and particularly in newly proliferated bone.

Furthermore, the very distinct line of demarcation between the yellow-gold induced fluorescence observed in areas of new bone formation and the bluish-gray autofluorescence in the matrix of the zone of vesicular cartilaginous hypertrophy likewise tend to militate against the view that localization in bone occurs solely in consequence of the availability of tetracycline in body fluids. Similarly, persistence of the induced fluorescence in bone concomitant with disappearance in other tissues, including cartilage, suggests that physicochemical phenomena other than diffusion alone are involved.

The observation (*2*) that one of the tetracyclines can form complexes with calcium at physiologic *p*H's makes it attractive to speculate that when tetracycline reaches a bone surface complex formation occurs between the fluorophor and calcium which effectively "fixes" the fluorescent material in bone. This would offer a reasonable explanation for both the rapidity of appearance of induced fluorescence following parenteral administration of tetracycline and its persistence in bone, as compared with cartilage and other soft tissues.[11] The fact that the observations noted here are virtually identical with those made by Cameron (*3*) with alizarin and by Arnold *et al.* (*4*) with radiocalcium supports such a view. On the other hand, data presented by Robinson and Cameron (*5*), indicating that distinct differences in tissue ultrastructure exist between the matrix and crystal patterns of bone and calcifying matrix in regions corresponding to those found, in this study, to present induced fluorescence and autofluorescence, respectively, suggest that a specific type of matrix is necessary for complex formation to occur between bone and the fluorophor. This could be either a direct binding of the fluorophor to matrix *per se* or a more complex interfacial interaction involving all 3 components—new bone matrix, calcium, and the fluorophor.

Irrespective of the chemical details of the binding mechanism, however, the findings presented here appear to be in complete consonance with the generalizations drawn by Neuman and Neuman (*6*) that surface reactions are the predominant types of biochemical reaction known to occur in bone and that the rates of these reactions are greater ". . . the lower the density, the greater the hydration, the newer the crystals and the better the fluid exchange." The hypothesis is tentatively offered, therefore, that tetracycline (or possibly a metabolic derivative with similar fluorescent properties in ultraviolet light) is rapidly distributed through the extracellular fluid compartment after administration and is capable of being bound, for relatively long periods of time, into regions of new bone proliferation, presumably by one or another type of surface reaction. This phenomenon appears to be characteristic of the 4-ringed naphthacenecarboxamide nucleus of tetracycline itself and to be unaltered by substitutions of either a chlorine atom in the 7 position or of a hydroxyl group in the 5 position.

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[11] The virtually instantaneous appearance of the fluorophor in tissue suggests that fluorescence may occur primarily and without the concomitant appearance of an activator system within the tissues, though the existence of the latter remains an unproved possibility.

Summary and Conclusions

Tetracycline, chlortetracycline, and oxytetracycline were administered to several species of laboratory animals. In ultraviolet light a brilliant yellow-gold induced fluorescence was apparent in diffusely distributed tissues, with the exception of the brain, almost instantaneously after intravenous administration and within 30 minutes after intraperitoneal injection. This induced fluorescence disappeared from all tissues except bone within 6 hours after a single parenteral injection. Bone fluorescence persisted throughout the 10-week period of observations following a single parenteral dose and could be detected even when remarkably small doses (0.3 mg./kg. tetracycline) were administered. Microscopically, localization in bone appeared to be limited to regions of new bone proliferation. It is suggested that bone fluorescence following tetracycline administration may be attributed to the binding of either the unaltered compound or, perhaps, a metabolic derivative to calcium and/or the matrix of newly formed bone.

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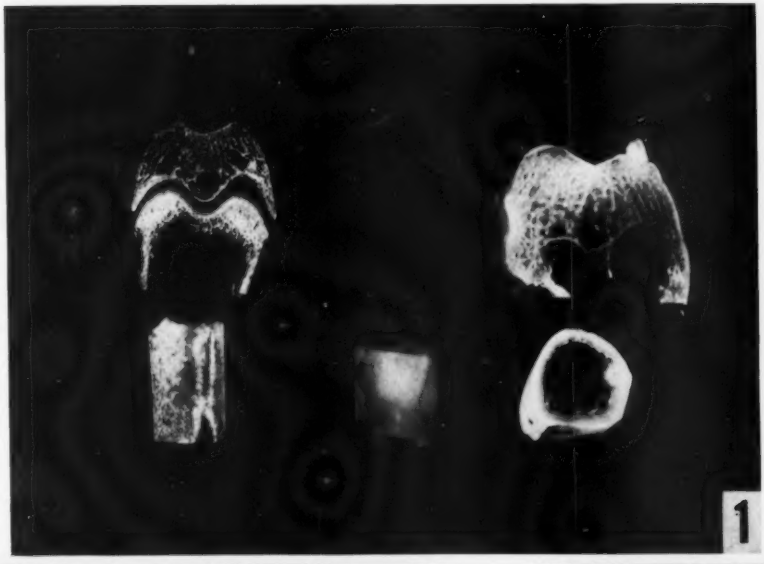
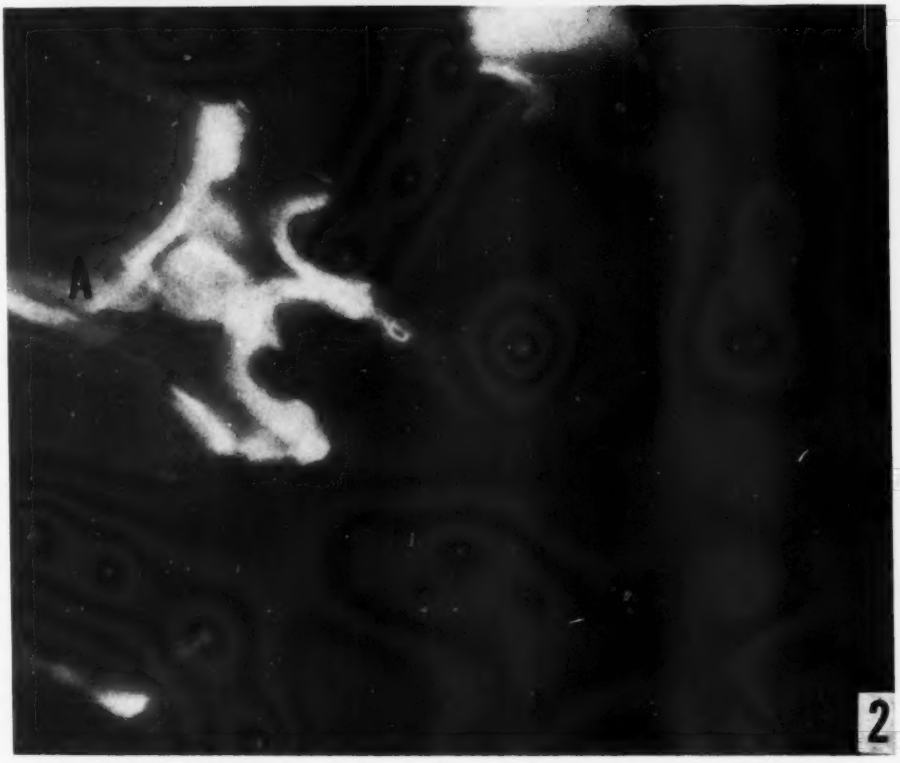
PLATE 15

FIGURE 1.—Localization of tetracycline in young bone (*left*) and adult bone (*center* and *right*) of rabbits. Lower end of the femur and femoral shaft 24 hours after a single (50 mg./kg.) intraperitoneal dose of tetracycline. In the young animal, the epiphyseal cartilage is grossly entirely free of the fluorophor, as is the zone of provisional calcification (gray region in this photograph). The immediately subjacent area of newly proliferating bone, however, fluoresces intensely yellow-gold in ultraviolet light. Bluish autofluorescence only is emitted from all regions of the control bone (*center*). In the adult rabbit, a cream-colored fluorescence, distinctly less intense than that observed in the young animal, is more or less uniformly distributed throughout the bone. Both the intensity of the emitted light and its distribution differ distinctly with the age of the animal.

FIGURE 2.—Low-power (80×) ultraviolet fluorescence photomicrograph demonstrating localization of fluorescent material in the capital femoral epiphysis of a rabbit weighing 1 kg. The animal had been given a single intraperitoneal dose of tetracycline (50 mg./kg.) and was sacrificed 24 hours thereafter. (A) shows 2 apposing bone trabeculae. The white areas, in this photograph, fluoresced intensely yellow-gold and the darker areas of the bony trabeculae showed bluish autofluorescence. The sharp line of demarcation between new and old bone and between bone and cartilage is quite evident. (B) is the region of vesicular cartilaginous hypertrophy and fluoresced a light gray-blue. (C) is the proximal cartilaginous portion of the epiphyseal plate and showed deep-blue autofluorescence.





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Permanent Modification (Mutation?) of a Histocompatibility Gene in a Heterozygous Tumor [1,2]

EVA KLEIN, GEORGE KLEIN, *and* LÁSZLÓ RÉVÉSZ,[3]
Institute for Cell Research and Genetics, Karolinska Institutet, Stockholm, Sweden

As one step in a program of studies on the occurrence, types, and causes of intercellular variability in neoplastic cell populations, an experimental system was described in a previous paper (*1*) that would detect and characterize specific gene mutations at the histocompatibility-2 (*H-2*) locus of mouse tumor cells. A similar system has been proposed by Lederberg (*2*) and used by Mitchison (*3*). We have carried out some model experiments (*1*) which showed that the system indeed possessed the expected unique qualities of specificity and sensitivity, that is, it could identify and selectively concentrate cellular variants that differed from the original type only with respect to the allelic substitution at a single gene locus. The variants were detectable even in cases where they were present as an extremely small fraction of the total population and in spite of the fact that they were intermixed in a random fashion with an overwhelming preponderance of cells of the original type that were being destroyed simultaneously by a selective host reaction. Surprisingly enough, it was found that some factor(s) involved in this process of destruction actually stimulated the growth of the surviving variants.

Essentially the system is based on the dependence of the basic relationship between transplanted tumor cells and their hosts, usually referred to as compatibility, upon the actual allelic substitutions at certain gene loci (histocompatibility genes) in the cells of both the tumor and the host. By suitable choice of donor and recipient mouse genotypes, it is possible to create situations in which a specific genetic change that occurs in only one or a few tumor cells may confer a high selective advantage of the host. By suitable choice of donor and recipient mouse genotypes, it is possible to create situations in which a specific genetic change that occurs in only one or a few tumor cells may confer a high selective advantage upon its bearer(s) and thereby become detectable. With the isogenic resistant lines (IR lines) of Snell (*4*), the changes studied can be pinned down to a single gene locus, *H-2*, which is perhaps the strongest and

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certainly the best known among the histocompatibility genes of the mouse (*5*). Our procedure has been to use tumors originating in F_1 hybrid mice produced by crossing 2 isogenic resistant lines. Since both parental lines have the same isogenic background, such hybrids are homozygous, at least theoretically, with respect to all gene loci except *H-2*, in which the parental strains differ, making the hybrids heterozygous. As a rule, neoplasms that originate in hybrids of this type do not grow progressively in either of the parental strains, since each parental strain is capable of reacting against the isoantigenic products of the foreign *H-2* allele derived from the other parental strain and both alleles are present in the tumor cells. Such tumors can be maintained only by transplantation to F_1 hybrids having the same genetic constitution as the animal in which they arose. However, if a specific mutation occurs at 1 of the 2 *H-2* loci in a diploid tumor cell that proceeds in the direction of the noncorresponding parental type (or, alternatively, one that renders the gene isoantigenically inactive for the noncorresponding parental strain), it will confer an absolute selective advantage upon the bearer whenever the cell population is tested in that particular parental strain, provided that the homograft reaction destroying the unchanged and incompatible cells does not also destroy the mutant in a nonspecific way. Our model experiments indicated (*1*) that the latter possibility can be dismissed and that the prospects for demonstrating specific mutations in this system are favorable.

The possibility of detecting specific mutations is not limited to those that proceed in the direction of one of the parental types. Since several parallel isogenic resistant lines of a number of mouse strains have been made available by Snell (*6*), each containing different histocompatibility genes on an isogenic background, it is theoretically possible to detect certain mutations that proceed in the direction of an unrelated allele. Suitable hosts for such studies can be produced by crossing the unrelated isogenic resistant line with one of the parental types of the F_1 tumor, thereby limiting the difference between tumor and host to a single gene locus. Changes proceeding in the direction of the foreign histocompatibility factor thus introduced may then become identifiable.

The present paper reports some observations on systems of this type. At least one permanent modification has been identified and characterized. Genetic and serologic evidence strongly indicated that it represents a specific mutation at the *H-2* locus proceeding in the direction of one of the parental types.

Materials and Methods

Mice

Four isogenic resistant sublines of mice were used, all of which had been developed by G. D. Snell (*4–6*), who kindly sent us breeding pairs in March, 1955. All 4 lines have a strain A background but differ with respect to the allele they carry at the histocompatibility-2 (*H-2*) locus. The original A/Sn line is genotypically $H\text{-}2^aH\text{-}2^a$, while the other 3 sublines,

A.SW, A.BY, and A.CA are *H-2^{s}H-2^s*, *H-2^{b}H-2^b*, and *H-2^{f}H-2^f*, respectively (*6*, *7*, and personal communication). The isoantigenic complex determined by each of these *H-2* alleles, with the exception of *H-2^f*, has been studied (*8*) and is currently denoted by a series of letters, each corresponding to an antigenic component. Thus *H-2^a* is antigenically CDEFK, *H-2^s* is C"S"EFk, and *H-2^b* is cD^{b}EFk.

The original A/Sn line was derived from the Strong A strain, established in 1921. According to the "Standardized Nomenclature for Inbred Strains of Mice" (*9*), it had been carried through 87 generations of inbreeding until 1952. The A/Sn subline was in its 19th generation when we received it and is currently in the 23rd brother × sister generation. The isogenic resistant lines were in their 24th (A.SW), 17th (A.BY), and 18th (A.CA) generations when they were received and are now in their 31st, 23rd, and 25th generations, respectively. From the 12th generation onward they were carried by continuous single-line brother × sister mating.

Animals of both sexes were used for the experiments at an age of 2 to 3 months. All mice were earmarked at weaning, each genotype having a different mark. Breeding mice were earmarked individually. The animals were kept on a standard diet in pellet form which, with drinking water, was available *ad libitum*.

Tumors

At the age of about 1 month, F_1 hybrid mice, produced by crossing the A/Sn strain with its isogenic resistant subline A.SW, were given 1 mg. methylcholanthrene dissolved in trioctanoin in 0.1 ml. injections deposited into the musculature of the right thigh. Three to 4 months after treatment, a number of animals developed sarcomas. Tumors that showed no signs of ulceration were removed under aseptic conditions. One part of the tissue was fixed for histologic examination, another part was frozen in our tumor bank, maintained at −79° C., (*10*) and the rest of the material was inoculated into mice of various genotypes. No tumors were carried serially over longer periods of time. Our aim was to study the immunogenetic behavior of these neoplasms while they were still as close to their original condition as possible, which was readily achieved with the help of the bank of frozen tumor tissue. Tumors of the first transfer generation were frozen in several parallel tubes. Whenever the supply of the various mouse genotypes permitted a test, 1 tube was thawed and inoculated into (A × A.SW)F_1 mice, and the resultant tumors were tested. Subsequent tests were carried out by using the parallel tubes until the supply was exhausted. Then a series of tubes from the second transfer generation was frozen and used for further tests. In this way the accumulation of serial passages was delayed considerably and no tumors were studied after more than 5 transfer generations. The method of freezing and thawing has been published elsewhere (*10*). We could find no evidence of any change in immunogenetic specificity after freezing and thawing with our method, which does not involve the addition of

glycerine or artificial solutions of any kind to the solid piece of fresh tumor tissue. Nor could we demonstrate the operation of any selection pressure as a result of our procedure. The experiments dealing with this problem and with different freezing procedures will be published elsewhere.

Most experiments were carried out with the MSWB tumor, a polymorphous spindle-cell sarcoma that arose in a 129-day-old male (A × A.SW)F_1 mouse. A number of tests were also performed with 4 other very similar tumors, designated DSWB, MNSB, MSWC, and MSWG, and 1 tumor of strain A origin, designated S4A.

Prior to inoculation, solid tumors aseptically removed were freed from macroscopically necrotic areas and brought into suspension by pressing them through a 60-mesh stainless-steel screen into Ringer's solution. Roughly comparable amounts of suspended tissue were injected subcutaneously into the right flank or bilaterally. The developing tumors were followed by regular palpations. Animals killed by progressively growing tumors were considered positive, whereas mice that survived an observation period of 3 months without tumor development or whose tumor had regressed during this period were registered as negative.

In a number of experiments, aliquots of the tumor-cell suspension were X irradiated in pyrex centrifuge tubes that were placed at a distance of 290 mm. from the X-ray tube. Doses between 300 and 500 r were administered at a rate of 156 r per minute. The X rays were generated at 145 kv. and filtered by 1 mm. Al.

Serologic Methods

Antisera were obtained by repeated inoculations of tumor tissue, indigenous to a certain mouse strain or a certain F_1 hybrid, into isogenic resistant mice carrying *H-2* alleles foreign to the tumor. Four to 7 challenges were given at intervals of 10 to 15 days. The mice were bled from the retro-orbital sinus through a glass capillary. The amount of blood taken from each animal was 0.5 to 0.8 ml. Blood pooled from 2 or 3 mice was placed in a small pyrex centrifuge tube, allowed to clot at room temperature, and subsequently centrifuged at 800 × g for 15 minutes. Only fresh antisera were tested.

Absorption of the antisera was carried out by using a mixture of minced liver, kidney, and spleen, washed 3 times with Ringer's solution and packed by centrifugation. Equal volumes of tissue and antiserum 1:4 were incubated at 37° C. for 1 hour. One or 2 absorptions were usually sufficient. After incubation the mixture was centrifuged and the absorbed antiserum collected.

The agglutinating capacity of the antisera was tested in the system, containing dextran and human serum, described by Gorer and Mikulska (*11*). The dextran preparation used was Intradex, Salt Free (Glaxo), made up to 6 percent solution in 5 percent glucose. Human serum was obtained from the blood-supply center of the St. Erik Hospital in Stockholm. It was collected under aseptic conditions from healthy donors. Samples of human serum were incubated at 56° C. for 1 hour. Subse-

quently they were absorbed with washed mouse tissue by the procedures described above for antisera. The absorbed human sera were stored at −20° C.

Agglutination tests were carried out in the following way: Red cells, obtained by puncture of the retro-orbital sinus, were washed twice in 0.85 percent saline and suspended to a 2 percent concentration in a 1:1 mixture of human serum and saline. Antiserum dilutions were made with dextran solutions, diluted to 2 percent with saline. Controls contained dextran, human serum, and mouse red-cell suspensions but no antiserum. The total volume in the agglutination tubes was 0.05 ml., consisting of 0.025 ml. diluted antiserum in dextran or, in the controls, dextran alone, and 0.025 ml. erythrocyte suspension. The mixtures were incubated at 37° C. for 1 hour. Agglutination was read exactly as described by Gorer and Mikulska (*11*).

The cytotoxic activity of the antisera was tested according to the procedure of Gorer and O'Gorman (*12*). Cell suspensions were made from pooled spleen, perfused with saline before use, thymus, and lymph-node tissue. The tissues were minced to a fine pulp and suspended in Ringer's solution and the suspension was stored in the refrigerator for 15 minutes to sediment large cell clumps. Subsequently the supernatant was pipetted off; it usually contained 10^6 to 10^7 cells per ml. With Schrek's eosin test (*13*), 85 to 95 percent of the cells remained unstained after mixing with Tyrode's solution containing eosin at a concentration of 1:2000. Equal volumes of cell suspension, antiserum (or normal mouse serum in the controls), and guinea-pig serum were incubated for 60 minutes at 37° C. Subsequently, the suspension was diluted 1:20 with eosin-Tyrode and the number of stained and unstained cells was rapidly counted in a Buerker hemocytometer. In the absence of specific antiserum, the percentage of eosin-unstained cells did not change to any detectable degree, while active antisera brought about highly significant changes in favor of the stained cells.

Results

Growth of Sarcoma MSWB in Various H-2 *Genotypes*

Sarcoma MSWB was induced in an (A × A.SW)F_1 hybrid mouse. Such an animal is theoretically homozygous with respect to all histocompatibility genes except *H-2*, which presents the heterozygous condition *H-2*^{a}*H-2*^{s}. According to the genetic laws of homotransplantation, MSWB would be expected to grow progressively in 100 percent of similar F_1 hybrids, but it would regress regularly in both parental strains. Fifty percent of the F_2 and backcross hybrids are heterozygous, and one half of such animals would therefore be expected to grow the tumor.

A series of samples derived from the first 5 transfer generations of sarcoma MSWB were tested experimentally. Part of the material was derived from tumors recovered after various periods of storage in our frozen-tumor bank (*10*). Since there was no systematic difference between different transfer generations or between tumors recovered after

frozen storage and tumors carried exclusively *in vivo*, the results of all experiments were pooled; they are shown in table 1, columns 3 and 4. It appears that the observations on transplantability of the sarcoma were in accord with our expectations, with one important exception. The tumor grew progressively in 100 percent of the F_1 hybrids of the type of origin and in 0 percent of the parent strain A, as expected. However, it was found that the tumor grew progressively in 11 of 63 animals of the A.SW parent strain, a result that was unexpected. The percentage of takes in F_2 and backcross hybrids was close to the theoretical 50 percent. As a rule, the tumor did not grow in animals carrying the unrelated *H-2*^{b} and *H-2*^{f} alleles, with the exception of 2 of 21 (A $\times$ A.BY)F_1 hybrids. One of the latter 2 tumors was tested again in (A $\times$ A.BY)F_1 mice and in other genotypes (*cf.* ‡ footnote to table 1) and behaved quite similarly to the original tumor; it grew in (A $\times$ A.SW)F_1 mice but not in other genotypes.

Concurrently with some of these experiments, part of each cell suspension used for inoculation was irradiated *in vitro* with 300 or 500 r and subsequently was tested in a similar manner (table 1, columns 5 and 6). The percentage of takes in A.SW mice rose to 24 of 49, *i.e.*, from 17 to 49 percent; the difference is highly significant. No takes were obtained in the A strain.

A number of the tumors that appeared in A.SW mice, inoculated either with the irradiated or the control tumor, were further tested in A.SW mice and other genotypes (table 1, columns 7 and 8). Since there was no systematic difference between tumors from the irradiated or control lines and several tests gave essentially the same result, the results of these experiments are also pooled. For clarity, all tumors that grew in A.SW and were subsequently tested or carried further will be called *variants*. The variants grew in 91 percent (133 of 146) of A.SW mice, a highly significant increase, when compared with either the control or the irradiated tumor. The percentage of takes in F_2 mice increased to 69 percent and in backcross mice to 93 percent. The variant tumors were consistent in their failure to grow in the other parental strain, A, or in genotypes carrying foreign alleles of *H-2*, thereby demonstrating that they did not become "nonspecific."

The behavior of the variant tumor might be explained by assuming that the *H-2*^{a} allele was lost from the tumor cells or that it mutated to *H–2*^{s} or to some third, indifferent form that lacked isoantigens D and K. The ideal percentage of takes would then be 100 percent in A.SW, 75 percent in F_2, and 100 percent in backcross mice. The actual values are only slightly different from those expected. In order to exclude other possible explanations, such as phenomena that imitate genetic changes, we designed additional experiments, which are described in the sections that follow.

Growth of Variant MSWB in Immunized A.SW Mice

Mutation or loss of the *H-2*^{a} gene are not the only possible mechanisms that may bring about changes of the type observed in the variant MSWB

tumor. It is conceivable that the *H-2*^{a} allele is still present in the nuclei of the variant cells, but they have changed with regard to their isoantigenicity, *i.e.*, their capacity to provoke the formation of anti-*H-2*^{a} antibodies in protective titers. Alternatively, their ability to resist such antibodies may have increased. Changes that are probably of this nature have been described (*14–17*), and one particular type, probably nongenetic in character, can be induced experimentally at will (*15–18*).

To reveal the presence of isoantigenic factors that would remain undetected in transplantation tests because of some mechanism of this kind, a more crucial experiment would be to test the tumor in preimmunized mice. Amos, Gorer, and Mikulska (*14*) have shown the efficiency of this method in detecting masked or latent groups of isoantigenic factors. In our work, the variant cells were tested for the presence of cellular receptors specifically sensitive to the action of preformed anti-*H-2*^{a} isoantibodies. This was done by inoculating the variant tumor into A.SW mice previously immunized against *H-2*^{a}. Immunization was accomplished by inoculating S4A, a tumor of strain A origin, or the original, unchanged line of MSWB. After temporary growth, these tumors regressed regularly in A.SW mice. One of several immunizing doses were given preceding the test with the variant tumor. In addition to immunized A.SW mice, a small number of (A $\times$ A.SW)F_2 hybrid mice that remained negative after inocultion with the ordinary MSWB tumor were also challenged with the variant tumor, after having been individually typed for the presence of *H-2*^{a} by the method of Gorer and Mikulska (*11*). The pooled results of these experiments are shown in table 2.

The variant tumor grew regularly in all immunized mice, with only 2 exceptions among 49 animals. The variant tumor was inoculated 10 to 14 days after the last immunizing challenge, with the exception of the experiment where 3 immunizing doses were given. In the latter instance, the original line of MSWB was used for immunization and, simultaneously with the third inoculation into the left flank, the variant tumor was injected into the right thigh. Seven of 8 mice developed progressively growing tumors in the right thigh only. In 1 mouse tumors appeared at both inoculation sites. This unusual finding can be explained by assuming that a variant cell(s) was included in the challenging MSWB inoculum. Disregarding this one instance, it can be stated that immunizing inocula of the original MSWB line regularly gave negative results, whereas the variant tumor was able to grow in immunized mice in an overwhelming majority of cases, when inoculated simultaneously with the last immunizing inoculum of the original line or 10 to 14 days thereafter.

The results of the serologic typing of the (A $\times$ A.SW)F_2 mice and of the transplantation tests were in accord. Since heterozygous mice of the constitution *H-2*^{a}*H-2*^{s} had previously died with MSWB tumors, the negative survivors were all homozygous. Three could be identified as *H-2*^{s}*H-2*^{s}; these were all susceptible to the variant tumor. Five mice were *H-2*^{a}*H-2*^{a} and proved resistant to the variant as well as the original tumor.

TABLE 1.—*Growth of original MSWB tumor line and its variants after inoculation into mice of various isogenic resistant lines and their hybrids, differing theoretically at the* H-2 *locus only**

Host	Genotype	MSWB control line—generations 1 to 5		MSWB after irradiation *in vitro* with 300 to 500 r		Variant MSWB, taken from occasional positive A.SW mice, inoculated with control or irradiated tumor	
		No. expts.	Pooled results†	No. expts.	Pooled results†	No. expts.	Pooled results†
1	2	3	4	5	6	7	8
A	*H-2^{a}H-2^a*	11	0/44 *0*	5	0/29 *0*	13	0/39 *0*
A.SW	*H-2^{s}H-2^s*	11	11/63 (**A**) *17*	5	24/49 (**B**) *49*	23	133/146 (**C**) *91*
(A × A.SW)F$_1$	*H-2^{a}H-2^s*	13	47/47 *100*	5	28/28 *100*	17	52/52 *100*
(A × A.SW)F$_2$	¼*H-2^{a}H-2^a* ½*H-2^{a}H-2^s* ¼*H-2^{s}H-2^s*	3	35/63 (**D**) *55*	1	9/19	3	68/99 (**E**) *69*
[(A × A.SW) × A.SW]BC	½*H-2^{a}H-2^s* ½*H-2^{s}H-2^s*	2	26/47 (**F**) *55*			3	69/74 (**G**) *93*
A.BY	*H-2^{b}H-2^b*	8	0/32 *0*	2	0/8 *0*	13	0/27 *0*
(A × A.BY)F$_1$	*H-2^{a}H-2^b*	6	2/21‡ *9*	1	1/3	5	1/15 *7*
(A.SW × A.BY)F$_1$	*H-2^{s}H-2^b*	1	0/7				

A.CA	*H-2^f/H-2^f*	2	0/7	1	0/3	13	1/27 *4*
(A × A.CA)F_1	*H-2^{a}H-2^f*	1	0/3	1	0/3	2	0/4

*Chi-square tests: **(A)** *versus* **(B)**: $\chi^2 = 12.7$ $P<0.001$
(A) *versus* **(C)**: $\chi^2 = 111.5$ $P<0.001$
(B) *versus* **(C)**: $\chi^2 = 42.1$ $P<0.001$
(D) *versus* **(E)**: $\chi^2 = 2.9$ $0.1>P>0.05$
(F) *versus* **(G)**: $\chi^2 = 24.5$ $P<0.001$
(D) *versus* expected value (50%): $\chi^2 = 0.8$ $0.5>P>0.3$
(E) *versus* expected value (75%): $\chi^2 = 2.1$ $0.2>P>0.1$
(F) *versus* expected value (50%): $\chi^2 = 0.5$ $P\sim0.5$

†Figures in roman type represent the number of mice killed by progressively growing tumors over the total number of inoculated animals; figures in italics are percentages.

‡One of the 2 tumors was tested in a number of genotypes with the following results: (A × A.BY)F_1—0/5; (A × A.SW)F_1—5/5; A.SW—0/4; A—0/5; A.BY—0/5; (A.BY × A.SW)F_1—0/3.

TABLE 2.—*Growth of the variant tumor in* H-2^s*-immunized A.SW mice*

Host	Genotype	Immunizing tumor	Original genotype of immunizing tumor	No. immunizing doses*	No. immunized mice killed by progressively growing variant tumor/Total no. inoculated
A.SW	*H-2^{s}H-2^s*	S4A	*H-2^{a}H-2^a*	1	17/18
A.SW	*H-2^{s}H-2^s*	MSWB	*H-2^{a}H-2^s*	1	11/12
A.SW	*H-2^{s}H-2^s*	MSWB	*H-2^{a}H-2^s*	2	8/8
A.SW	*H-2^{s}H-2^s*	MSWB	*H-2^{a}H-2^s*	3	8/8†
(A × A.SW)F$_2$	*H-2^{s}H-2^s* (serologically typed)	MSWB	*H-2^{a}H-2^s*	1	3/3
(A × A.SW)F$_2$	*H-2^{a}H-2^a* (serologically typed)	MSWB	*H-2^{a}H-2^s*	1	0/5

*If several challenges were given, they were administered at intervals of 10 to 14 days. With the exception of the experiment in which 3 immunizing doses were given, the variant tumor was inoculated 3 to 5 weeks after the last dose. In the experiment where 3 immunizing doses were given, the variant tumor was inoculated simultaneously with the last dose but at a different site.

†One of the 8 mice developed 2 tumors after the third challenge, given simultaneously with the inoculation of the variant tumor. One of these tumors was located subcutaneously in the left flank, the site of the third immunizing dose with the original line of MSWB, while the other appeared in the musculature of the right thigh, the site of inoculation of the variant tumor. Both tumors grew progressively until the death of the animal.

Serologic Tests

The variant tumors were tested for the presence or absence of the specific isoantigens of the *H-2^a* group and were compared to the original MSWB tumor by two methods. In one series of experiments, immune sera were produced in isogenic resistant mice of the genotypes *H-2^{b}H-2^b* or *H-2^{f}H-2^f* by repeated inoculations of the original or the variant tumor. The sera were compared with respect to their agglutinating and cytotoxic activities against red cells and lymphocytes, of *H-2^{a}H-2^a* and *H-2^{s}H-2^s* origin, respectively. The isoantigenic system determined by the *H-2^a* allele (CDEFK, *8*, *19*) has 3 components in common with the isoantigenic system determined by *H-2^s* (C"S"EFk), but two components are different. To demonstrate the presence of isoantibodies directed specifically against *H-2^a* it is therefore necessary to remove components anti-C, anti-E, and anti-F and to look for anti-D and anti-K. This was done by absorbing the sera (*H-2^{s}H-2^s*) with mixtures of liver kidney, and spleen from A.SW mice. Altogether 8 series of experiments were carried out. Since they all gave similar results, only 1 series, which is typical, is given in table 3, which shows the effect of isoimmune serum, produced in isogenic resistant A.BY (genotype *H-2^{b}H-2^b*) or A.CA (genotype *H-2^{f}H-2^f*) mice by repeated challenges with the regular (genotype *H-2^{a}H-2^s*) or the variant (presumed genotype *H-2^{s}H-2^s*) MSWB tumor, when tested against the red blood cells and lymphocytes of A (genotype *H-2^{a}H-2^a*) and A.SW (genotype *H-2^{s}H-2^s*) mice.

The regular line of MSWB elicited the formation of hemagglutinating and cytotoxic antibodies against both *H-2^a* and *H-2^s*. Activity directed against strain A cells could not be removed by absorption with A.SW tis-

TABLE 3.—*Effect of isoimmune serum from A.BY and A.CA mice on red cells and lymphocytes of A and A.SW mice**

Cells from mouse strain	Antiserum			Effect on red blood cells at following antiserum dilutions:								Control (no antiserum)	Effect on lymphocytes after 1 hour's incubation with antiserum compared with controls (*percent survivors*)
	Source	MSWB line used for immunization	Absorbed with	4	8	16	32	64	128	256	512		
A	A.BY	Regular		±	+	++	++	++	++	++	++	−	5
A		Variant		+	++	++	++	++	+	+	+	−	54
A.SW		Regular		±	++	++	++	+	+	±	−	−	4
A.SW		Variant		±	±	+	++	++	+	−	−	−	5
A	A.BY	Regular	A.SW liver, kidney, spleen	+	+	++	++	++	+	+	+	−	9
A		Variant	" " " "	−	−	−	−	−	−	−	−	−	102
A.SW		Regular	" " " "	−	−	−	−	−	−	−	−	−	105
A.SW		Variant	" " " "	−	−	−	−	−	−	−	−	−	102
A	A.CA	Regular		+	+	++	++	++	++	++	+	−	29
A		Variant		++	++	++	++	++	++	++	+	−	88
A.SW		Regular		+	++	++	++	++	++	++	++	−	8
A.SW		Variant		+	+	++	++	++	++	+	+	−	6
A	A.CA	Regular	A.SW liver, kidney, spleen	++	++	++	++	++	++	++	−	−	56
A		Variant	" " " "	−	−	−	−	−	−	−	−	−	100
A.SW		Regular	" " " "	−	−	−	−	−	−	−	−	−	90
A.SW		Variant	" " " "	−	−	−	−	−	−	−	−	−	98

*Five to 7 immunizing inoculations were given at intervals of 10 to 15 days.

sue, which shows that it was specific for *H-2*^{a}. In contrast, isoimmune sera produced against the variant line of the tumor were consistently lacking in specific anti-*H-2*^{a} activity. They did show activity against red cells of A origin and, to a somewhat smaller extent, against lymphocytes, but this activity could be removed completely by absorption with A.SW tissue and, thus, was due to the isoantigenic components that *H-2*^{a} and *H-2*^{s} have in common. These findings indicate that the variant tumor has lost the antigenic components specific for *H-2*^{a}, *i.e.*, D and K.

In another series of experiments the regular and the variant tumors were compared for their capacity to absorb anti-D and anti-K activity from an anti-*H-2*^{a} serum. The antiserum was produced by immunizing A.SW (*H-2*^{s}*H-2*^{s}) mice against the ascitic form of adenocarcinoma TA3, which originated in strain A (*H-2*^{a}*H-2*^{a}) mice and its activity was tested against strain A cells. The results of 2 typical experiments are shown in table 4. While the original line removed both the agglutinating and the cytotoxic activity of such sera, the variant line consistently failed to do so. This confirms the conclusion that the variant tumor has lost isoantigens D and K.

Experiments with Other Tumors

Some experiments were also carried out with a few other methylcholanthrene-induced tumors. As a rule, the histology of these neoplasms was quite similar to that of MSWB. Those experiments that were carried out on a sufficient number of animals up to the time of the present writing are summarized in table 5. Three F_1 tumors—DSWB, MNSB, and MSWG—behaved in accordance with the genetic laws of transplantation; they grew exclusively in F_1 hybrids genetically identical with the mouse in which they had originated but not in any of the parental strains. DSWB and MNSB were also tested on a small number of mice after irradiation. With the limited material available no change could be detected after this treatment.

Different behavior was exhibited by sarcoma MSWC, which is of similar histologic and genetic origin. From its earliest history this tumor violated the *H-2* barrier extensively in that it was able to grow in a proportion (although never in 100 percent) of both parental strains and also in isogenic resistant mice carrying foreign alleles at the *H-2* locus. Irradiation did not cause any apparent change in this behavior. It has not been possible to select precise variant sublines from this tumor thus far. Serial testing of tumors that appeared in A.SW mice did not reveal any increased tendency to grow in A.SW that was concomitant with a decreased tendency to grow in A mice; the behavior of such tumors was similar to that of the original neoplasm.

One tumor of strain A origin (S4A) was also tested for growth in A.SW mice under control conditions and after irradiation. The tumor failed to grow in the foreign genotype in both cases.

TABLE 4.—*Comparison of regular (genotype* H-2^{a}H-2^a*) and variant (presumed genotype* H-2^{a}H-2^o*) MSWB tumor for ability to absorb agglutinins and cytotoxic antibodies from anti-*H-2^a *isoimmune sera* *

Antiserum	Effect on red blood cells of A mice at following antiserum dilutions:								Control (no antiserum)	Effect on A lymphocytes after 1 hour's incubation compared with controls (*percent survivors*)
	4	8	16	32	64	128	256	512		
1. A.SW anti-A										
Unabsorbed	+	+	++	++	++	++	++	++	−	8
Absorbed with the regular line of MSWB	±	−	−	−	−	−	−	−	−	93
Absorbed with the variant line of MSWB	+	++	++	++	++	++	±	−	−	16
2. A.SW anti-A										
Unabsorbed	−	±	++	++	++	++	++	++	−	23
Absorbed with the regular line of MSWB	−	−	−	−	−	−	−	−	−	97
Absorbed with the variant line of MSWB	+	+	++	++	++	++	±	±	−	18

*Four or 5 challenges were given at intervals of 10 to 15 days.

TABLE 5.—*Experiments with other induced tumors in isogenic resistant mice*

Tumor	Induced in	Genotype	Progressive growth in:*					
			(A × A.SW)F$_1$ (*H-2^{a}H-2^s*)	A (*H-2^{a}H-2^a*)	A.SW (*H-2^{s}H-2^s*)	A.BY (*H-2^{b}H-2^b*)	A.CA (*H-2^{f}H-2^f*)	(A × A.BY)F$_1$ (*H-2^{a}H-2^b*)
DSWB	(A × A.SW)F$_1$	*H-2^{a}H-2^s*	51/51	0/18 0/15†	0/15 0/20†			0/2
MNSB	"	"	20/20	0/17 0/5†	0/8 0/6†	0/5		
MSWC	"	"	75/75	29/35 3/8† 4/12‡	61/78 4/11† 3/8‡	10/16	6/9	5/7
MSWG	"	"	8/8	0/12	0/15			
S4A	A	*H-2^{a}H-2^a*		9/9 9/9†	0/18 0/18†			

*The figures denote the ratio of mice killed by progressively growing tumors to the total number of inoculated animals.
†After irradiation of the inoculum with doses between 300 and 500 r *in vitro*.
‡After irradiation, as described in the preceding footnote, followed by 1 passage in A.SW mice.

Discussion

It is impossible to furnish direct proof by currently available methods that would be fully conclusive in demonstrating the occurrence of *gene* mutations in somatic cells—normal or neoplastic. The system used in the present study is indirect and does not distinguish between intra- and extrachromosomal factors. Nevertheless, a body of evidence has been obtained which strongly suggests that the variant cells of the MSWB sarcoma are indeed mutants, differing from the original cells by a single allele at the *H–2* locus. The following findings are relevant in this respect:

MSWB is a heterozygous tumor that originated in an (A $\times$ A.SW)F_1 mouse, genotypically *H-2*^{a}*H-2*^{s}. Its original line usually regresses in both parental strains, because each of them can react against the isoantigenic products of the foreign *H-2* allele derived from the opposite parental strain and contained in the tumor cells in a single dose. Model experiments (*1*) have demonstrated the extreme specificity and sensitivity of this reaction, *viz.*, its ability to distinguish between compatible and incompatible cells even if compatibility is a matter of a single gene difference and the compatible cells comprise a small fraction of the tumor-cell population. The *variant* line of MSWB was recovered from occasional takes in *one* of the parental strains—A.SW. In contrast to the original line, this variant grew regularly in A.SW mice on further tests but consistently failed to grow in the other parental strain, A, or in other isogenic resistant lines carrying unrelated *H-2* alleles. This indicated that the progenitor(s) of the variant cells might have mutated from *H-2*^{a}*H-2*^{s} to *H-2*^{s}*H-2*^{s} or, alternatively, that it (they) might have lost the *H-2*^{a} gene. Still another possibility was that *H-2*^{a} was still there but that some other change had occurred which affected the structure of the cell surface, for example, and prevented the release of isoantigens in sufficient quantity to elicit the formation of a protective titer of isoantibodies. Such apparent "antigenic simplifications" have been described and could be unmasked by the use of mice preimmunized against the isoantigenic factors produced by the particular *H*-loci in question (*14*). These studies led to the conclusion that, in the cases studied, it seemed ". . . unlikely that any antigens have been lost, although their relative proportions may have been radically altered."(*14*). In order to decide whether we were dealing with a similar case, we tested the variant line in A.SW mice that had been immunized previously against the original line. Such mice were killed regularly by progressively growing tumors, in spite of the fact that the original tumor had regressed previously in the same animals 1, 2, or 3 times. This suggests that we are dealing with a change that is different in nature from the one studied by Amos, Gorer, and Mikulska (*14*).

Another means of demonstrating the absence of the specific isoantigens of the *H-2*^{a} group in the variant cells was made possible by the serologic methods developed by Gorer and his associates (*8, 11, 12, 19*). In one series of experiments, isogenic resistant mice carrying the foreign *H-2*^{b} or *H-2*^{f} alleles were hyperimmunized against the original and the variant tumor, respectively. Their sera were found to contain both hemagglutin-

ins and cytotoxic antibodies against *H-2*^{s} cells. Specific anti-*H-2*^{a} antibodies could be demonstrated regularly in the sera of mice immunized against the original tumor but were always absent from the sera of mice immunized against the variant tumor.

In another series of experiments the original and the variant tumors were compared for their capacity to absorb specific anti-*H-2*^{a} antibodies from hyperimmune sera produced by immunizing A.SW mice with an ascites tumor of strain A origin (adenocarcinoma TA3). Both hemagglutinins and cytotoxic antibodies were absorbed regularly by tumors of the original line, whereas the variant line failed to show any activity.

Taken together, these results suggest very strongly that specific isoantigenic products of the *H-2*^{a} gene, components D and K, are absent from the variant cells. The most plausible explanation of these findings is loss or mutation of the *H-2*^{a} allele itself.

The variant tumor was recovered repeatedly from positive A.SW mice inoculated with the original line, a few of which were found in a number of tests made with samples of inoculum taken from different transfer generations of the original tumor. The question arises whether all these variants represent independent mutations or whether we are dealing with one or a few variant clones already present in the original line with low or moderate frequency at an early stage of its history. Since the variants could be recovered as early as the first transfer generation of the original tumor and they did not show any striking increase in frequency during the 4 subsequent transfer generations, it seems probable that we are dealing with a single clone, or perhaps a few clones, rather than with a large number of independent mutations. This is also suggested by the fact that the change has always proceeded in the direction of only *one* parental type (A.SW) and never in the direction of the other (A). In 3 other tumors of similar origin—DSWB, MNSB, and MSWG—that were carried and tested in a similar way, although less extensively, no corresponding changes could be detected, *i.e.*, the tumors consistently failed to grow in both parental strains. This might be taken to suggest that the change demonstrated in MSWB is a rare event that occurred in this tumor by chance once or perhaps a very few times and the apparent repetition is due to the same clone(s). Alternatively, there may be some special cytologic abnormality in MSWB that makes cells of this tumor particularly liable to lose *H-2*^{a} but not *H-2*^{s}. This possibility cannot be eliminated from consideration, particularly since the frequency of variant MSWB tumors increased significantly when the inoculum was pretreated *in vitro* with low doses of X rays. It is conceivable that X rays may act by increasing the frequency of similar but independent changes leading to the loss of *H-2*^{a}. Alternatively, they may exert a selective effect, favoring pre-existent variant clones. This problem is now under investigation.

It seems that the homotransplantability of mouse tumors may change in a number of distinctly different ways. Some neoplasms, like MSWC in the present study or some of the tumors studied by Sachs and Gallily

(*20*), are regularly transplantable (although seldom 100 percent transplantable) to foreign genotypes in the very beginning of their history. Our tumor MSWC differs sharply from MSWB in the change observed in the latter, since it was not possible to select clear-cut sublines that would grow in 100 percent of 1 particular parental or foreign genotype and regress in other unrelated genotypes. The behavior of this tumor is not suggestive of distinct mutations in particular genes but recalls rather the behavior of many long-transplanted and "nonspecific" carcinomas and sarcomas. Such tumors were studied extensively by Hauschka and Levan (*21–23*), who demonstrated a correlation between the development of heteroploidy in cell populations and the loss of strain specificity. It is possible that the behavior of MSWC is attributable to a similar mechanism, distinctly different from simple mutation.

It is of interest to compare further our results with those of Sachs and Gallily (*20*). These authors tested 12 primary carcinomas and sarcomas. Eight tumors were strain specific and did not take, even occasionally, in foreign genotypes. Two tumors grew occasionally, but these could not be perpetuated by selective transfers. One tumor was nonspecific from the very beginning, and continued transplantation in a foreign genotype did not result in the selection of cells particularly suited for that genotype to the exclusion of others. Another tumor sometimes grew in a foreign mouse stock, and when transferred selectively, resulted in a line that grew in a large percentage of that stock. These types can also be recognized in our material. Their strictly strain-specific tumors are comparable to our tumors DSWB, MNSB, and MSWG. Their tumors that give occasional takes in a foreign genotype but fail to "breed true" in the form of specific variants after continued selective transfer can be compared to our MSWB sarcoma when tested in $(A \times A.BY)F_1$ hybrids. Their tumors that are nonspecific from the very beginning and lack the ability to develop into specific sublines with selective compatibility are comparable to our MSWC. Finally, the last category, comprising tumors available for the selection of variant lines, is comparable to our MSWB and its variant in A.SW mice. There is, however, one important difference between the R14 sarcoma of Sachs and Gallily (*20*), which represents this category, and our variant. R14 is a tumor of C3H origin. Its variant line was selected by serial transfer in Swiss mice. After this procedure, the line was able to grow in Swiss mice and, in addition, in a number of unrelated genotypes. On the other hand, the variant MSWB grows only in the specific genotype where it was selected. Swiss is a heterozygous stock, however, that may well contain several segregating alleles of *H-2*, and it is possible that the adaptation to such a mixed stock may involve the multiple selection of different genotypes. Although this is one possible explanation of the differences, it is equally possible that we are dealing with two different types of change.

The cellular mechanism of these different types of behavior remains hypothetical at present. Variation in MSWB represents, most probably, single gene mutations followed by selection. The case of MSWC and

other nonspecific tumors may depend on the profound remodeling of the entire chromosome complement, found by Levan (*23*) and by Yerganian (*24*) in other homotransplantable tumors. While these two mechanisms presumably depend on random variations followed by selection, there is a third, apparently quite different, mechanism, *viz.*, the induced modification brought about by the action of certain host environments. Barrett and Deringer (*15, 16*) described a regular change in the histocompatibility requirements of a mammary carcinoma of C3H origin after passage through F_1 hybrid mice, derived by outcrossing C3H with another, resistant strain. We have studied the mechanism of this change (*18*) and have found that it was not due to the selection of pre-existing variants but could be regarded as having been induced by some factor(s) present in the host environment of the F_1 mouse. Available evidence suggests that this represents a case of nongenetic modification that does not seem to involve a loss of isoantigens but rather an increased tolerance to certain isoantibodies. This view is based on our unpublished observation that the difference between the original and the altered line disappears if they are compared in preimmunized F_2 or backcross hybrids. Another difference between this adaptive modification and the presumably genetic changes discussed above is that while the latter may involve the strongest histocompatibility locus, *H-2*, the former is probably restricted to weaker isoantigenic systems. This is indicated by the observation that all tumors that have been found to undergo a modification of the Barrett-Deringer type have retained a minimum requirement of one histocompatibility factor. Probably this one factor corresponds to the *H-2* system; we have consistently failed to demonstrate any Barrett-Deringer effect upon passage of mammary carcinomas of strain A origin through $(A \times A.SW)F_1$ hybrids, heterozygous for the *H-2* locus only. This suggests that *H-2* is impermeable to modifications of this type.

In conclusion, it appears that the available evidence regarding variations in the histocompatibility behavior of different primary tumors and the various ways in which this behavior may change under the influence of selective or modifying new host environments, incomplete and fragmentary though it is, nevertheless reveals parts of an extremely complex and, indeed, protean picture. This is true even if consideration is restricted to neoplasms induced by the same carcinogenic agent in the same tissue of the same host genotype, as was the case with the experimental material of the present paper. It seems that variations exist between different tumors with regard to their primary behavior, the stability of the behavior, and, in cases of changeability, the possible pathways of change. It is tempting to speculate that these variations reflect differences in the cytogenetic makeup of the various neoplastic-cell lineages. Such differences may be secondary consequences of the same basic neoplastic transformation, where branching into different directions would occur as part of the evolution of the established tumors. It is equally possible, however, that neoplastic transformation may travel a number of pathways, not necessarily related, which lead to the same end

result—neoplasia—but are different with regard to the underlying cellular mechanism.

Summary

Sarcomas were induced by methylcholanthrene in F_1 hybrids of 2 isogenic resistant mouse strains, A and A.SW. Theoretically, these strains differ only in the allelic substitution at a single gene locus, *H-2* (histocompatibility-2). One would, therefore, expect the sarcoma cells to be generally homozygous, with the exception of the *H-2* locus, which presents the heterozygous condition $H\text{-}2^a H\text{-}2^s$. According to the genetic laws of homotransplantation, such tumors can be transplanted only to F_1 hybrids isogenic with the original host but not to the parental strains or to mice carrying foreign alleles at the *H-2* locus.

Three sarcomas behaved according to expectation. Two of them also were tested after irradiation *in vitro* prior to transplantation but showed no change. One sarcoma (MSWB) behaved similarly, except that it also grew in 17 percent of one of the parental strains, A.SW. Upon subsequent testing, the tumors that appeared in A.SW were found to differ from the original tumor and were therefore considered variants. They grew regularly in a large percentage of A.SW mice, even if these were immunized against the original line of MSWB by repeated inoculations. They failed to grow in the other parental strain, A, or in other isogenic resistant lines of the A strain carrying unrelated alleles at the *H-2* locus. While the original line provoked the formation of both anti-$H\text{-}2^a$ and anti-$H\text{-}2^s$ isoantibodies in isogenic resistant mice of the genotype $H\text{-}2^b H\text{-}2^b$ or $H\text{-}2^f H\text{-}2^f$, the variant tumors provoked only anti-$H\text{-}2^s$ and not anti-$H\text{-}2^a$. This was true for both hemagglutinins and cytotoxic antibodies. Anti-$H\text{-}2^a$ activity could be absorbed quantitatively from isoimmune sera by tumor tissue from the original line but not by the variant tumor. These findings suggest that $H\text{-}2^a$ mutated to $H\text{-}2^s$ or, alternatively, was lost from the cells of the variant tumor.

Irradiation *in vitro* with doses between 300 and 500 r prior to inoculation increased the yield of variant tumors from 17 to 49 percent. The possible mechanism of this action has been discussed.

A fourth tumor of similar morphologic and genetic origin (MSWC) grew in a large percentage of parental and foreign genotypes from the beginning of its history. It was not possible to select precise variants with selective compatibility from this tumor.

It seems that the histocompatibility requirements of different primary tumors of similar origin may vary and that such requirements may change by different mechanisms during the subsequent transplantation history of the various cell lines. Such mechanisms as specific mutation of histocompatibility genes, profound remodeling of the entire chromosome complement, and environmentally induced adaptations have been indicated to exist for different cases. Different tumors may show a different tendency to undergo the various types of modification. This can be taken as a probable indication of differences in their primary cytogenetic structure.

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A Method for Preparing Wound Washings and Bloody Fluids for Cytologic Evaluation[1]

SARA H. DEWITT, PASCO R. DEL VECCHIO, JACQUELINE I. BORELLI, *and* ALBERT W. HILBERG, *National Cancer Institute,*[2] *Bethesda, Maryland*

The identification of tumor cells in operative wounds has been carried out in this laboratory during the past 3 years. It was done in cooperation with the surgical staff of the National Cancer Institute in order to determine the amount of seeding of operative wounds by fragments of tumor tissue or individual tumor cells (*1*, *2*). One of the major difficulties in the cytologic examination of wound washings was that the tumor cells were masked by erythrocytes and fibrin. A method for preparing smears has been developed that obviates this problem. This method is also useful in preparing other body fluids, such as pericardial, pleural, and ascitic fluids, and vaginal and cervical smears that may contain blood.

Method

The unfixed specimen was placed in a conical glass tube and centrifuged at 2000 × g for 30 minutes. The supernatant was removed, and smears of the sediment were made on clean glass slides previously coated with 1 drop of Mayer's albumin-glycerol solution (*3*). The material was smeared thinly and evenly by placing approximately 2 drops of the sediment between 2 slides and gently separating them by sliding them apart. Thick preparations were unsatisfactory. The preparations were immediately placed in Coplin jars that contained modified Carnoy fixative consisting of a mixture of 70 cc. of 95 percent ethyl alcohol, 25 cc. chloroform, and 5 cc. glacial acetic acid. This fixing solution was stored in a tightly stoppered brown bottle at room temperature. A minimum of 30 minutes was required for proper fixation of the smears; however, they could be left for 48 hours or longer. The smears were then stained by the Papanicolaou technique. Fifteen seconds in undiluted Harris' hematoxylin solution, 30 seconds in "OG 6," and 45 seconds in "EA 50" gave good results (*4*). Other nuclear cytoplasmic stains, such as hematoxylin-eosin, may be used. After the staining was completed, the slides were mounted with a permanent mounting medium.

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Results

The method for the preparation of wound washings has been found satisfactory for removing erythrocytes and fibrin that interfere with visualization of the tumor cells in this material (fig. 1). The cytologic details of the significant cells are sufficiently preserved to enable critical evaluation (figs. 2, 3). In a comparison of over 100 cases in which the modified Carnoy technique and the routine ether-alcohol fixative were used, it was found that the modified Carnoy solution did not injure or destroy the tumor cells, yet the masking effects of the erythrocytes and fibrin are almost entirely removed. A modified Carnoy solution was found to be more satisfactory than the usual Carnoy solution (*5*) for fixing the smeared material since it gave better cytologic detail.

This method can be applied to any unfixed fluids, such as pericardial (figs. 4, 5), pleural, and ascitic fluids in which large amounts of erythrocytes and fibrin may obscure tumor cells. It may also be used for vaginal and cervical smears. The evaluation of smears is rendered more reliable by removing erythrocytes and fibrin, screening is less tiring, and bloody fluids which might otherwise have been considered useless for cytologic evaluation can be utilized.

Summary

A method of fixation based on a modified Carnoy solution has been found to remove satisfactorily erythrocytes and fibrin from wound washings and other body fluids that may contain blood, so that the tumor cells in the material may be visualized better. The method may also be used for vaginal and cervical smears.

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PLATES

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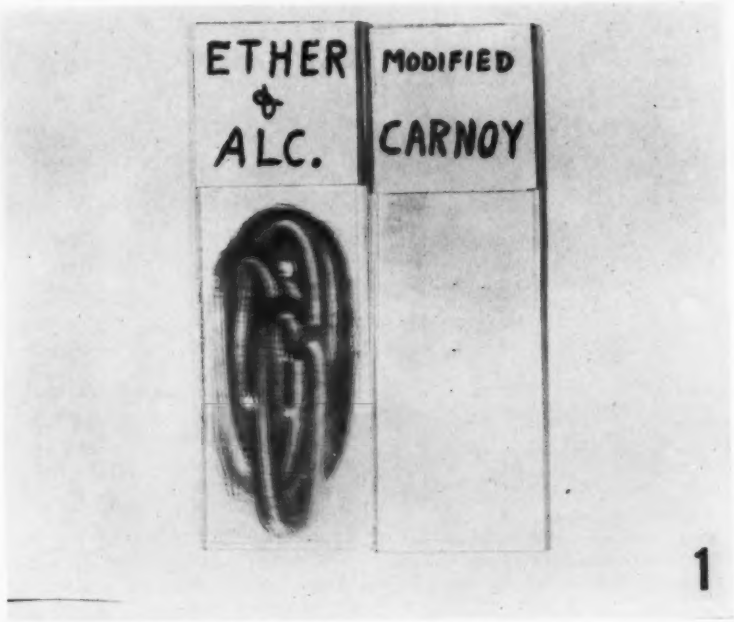
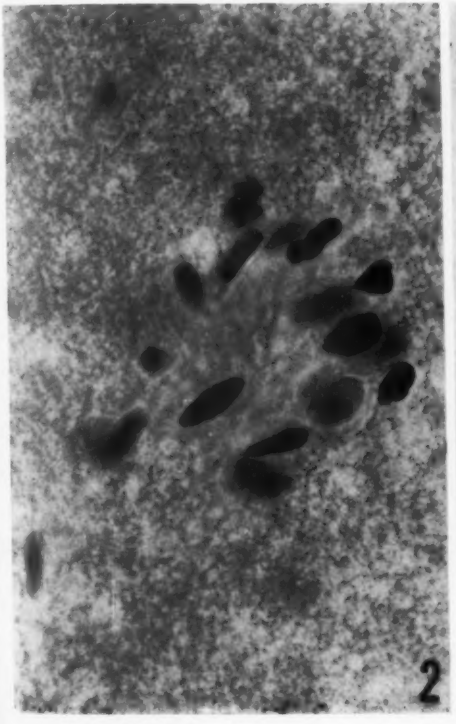
PLATE 16

FIGURE 1.—Gross comparison of smears prepared from sediment of bloody fluid fixed in ether-alcohol fixative and modified Carnoy fixative. Papanicolaou stain.

FIGURE 2.—Group of neoplastic cells (epidermoid carcinoma) from wound washing taken after biopsy, right lateral pelvic wall. Smear fixed in ether-alcohol. Papanicolaou stain. × 750

FIGURE 3.—From same case as figure 2. Group of neoplastic cells. Smear fixed in modified Carnoy. *Note* clarity of background and distinct cellular detail. Papanicolaou stain. × 750





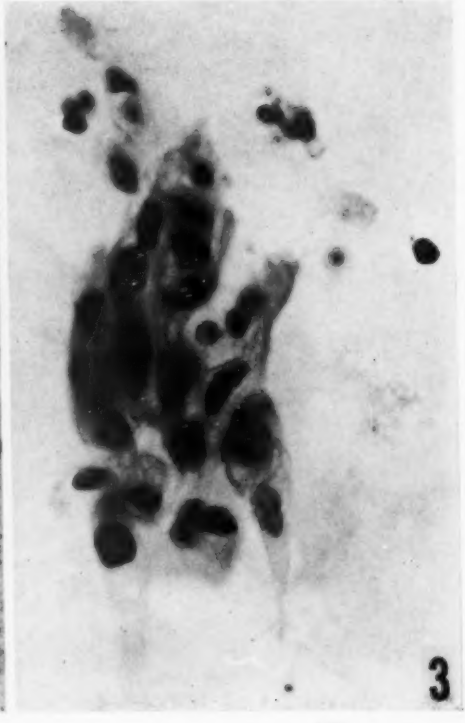


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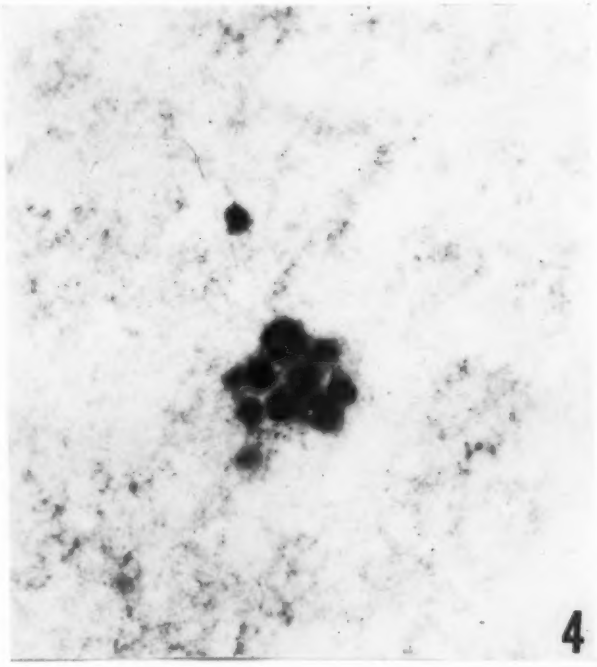
PLATE 17

FIGURE 4.—Group of neoplastic cells from pericardial fluid of patient with malignant melanoma. Smear fixed in ether-alcohol. Papanicolaou stain. × 700

FIGURE 5.—Group of neoplastic cells from the same case as figure 4. Smear fixed in modified Carnoy. *Note* clarity of background and distinct cellular detail. Papanicolaou stain. × 700



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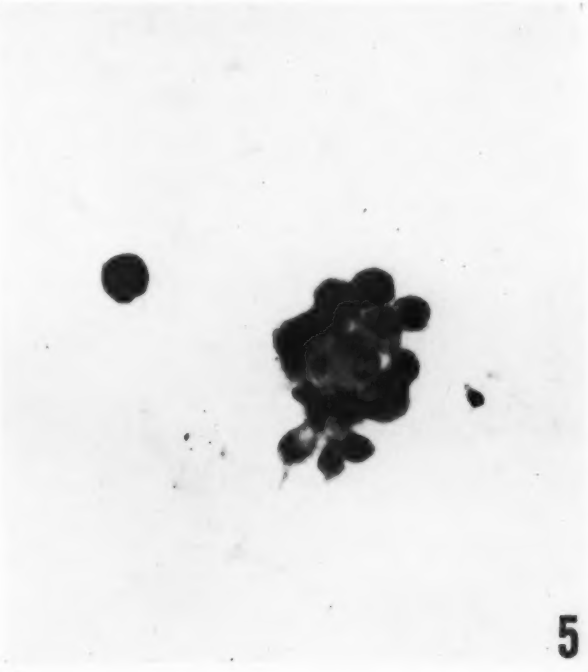

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Genetic Factors Influencing Irradiation Protection by Bone Marrow. I. The F_1 Hybrid Effect[1]

DELTA E. UPHOFF, *National Cancer Institute,*[2] *Bethesda, Maryland*

It has been shown that the inoculation of homologous or heterologous bone marrow protected mice against a lethal total-body dose of X radiation during the first 30 days post irradiation (*1*, *2*). It was observed later by Barnes and Loutit (*3*) that inbred mice irradiated and treated with homologous marrow survived only a relatively short time after the initial recovery from the radiation effects as compared with similar mice treated with isologous marrow.

In the present study, an attempt has been made to determine which known genetic laws of tissue transplantation govern the capability of normal mouse bone marrow to protect mice against a lethal dose of radiation. This is the first of a series of papers describing some of the phenomena encountered in this study.

It was originally hoped that this type of study might also help to determine the validity of the theory of "cellular repopulation" in irradiation protection as opposed to the theory of the "humoral factor" (*4*). The subsequent establishment of considerable evidence in favor of the theory of "cellular repopulation" (*5–7*) has emphasized the importance of genetic factors in protection by bone marrow following lethal X irradiation. The problem now appears to be one of tissue transplantation.

The basic genetic principles of tissue transplantation have been clearly defined and follow simple Mendelian laws of inheritance (*8*). The same principles apply to the transplantation of normal as well as neoplastic tissues. In the transplantation of tumors, for example, F_1 hybrids produced by crossing 2 inbred strains of mice will grow tumors progressively if the tumors are indigenous to either parental strain or to the specific F_1 hybrid. However, in the present experiments, it was found that when bone marrow of the parental strains of some selected F_1 hybrids was used to protect these hybrids against a lethal dose of X radiation, the protection was only transient. The mice died from the effects of a late irradiation syndrome. The inoculation of F_1 marrow into irradiated F_1 hybrids, however, afforded excellent protection under the same experimental condi-

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tions. The unexpected ability of the F_1 marrow to afford better protection than marrow from either of the parental strains has been called the "F_1 hybrid effect," and certain details will be described.

Material and Methods

Inbred strains and their F_1 hybrids.—Since studies of the genetics of tissue transplantation were made possible only through the development of highly inbred strains of mice, the strains used in the following experiments were from well-established, carefully inbred lines. They were either raised in this laboratory or were obtained from the colony of Dr. M. K. Deringer. The strain A/De mice originated from the Heston subline and have been maintained through 27 generations of brother × sister matings by Deringer. The strain BALB/c mice were obtained from Andervont and maintained by Deringer through 9 generations of brother × sister matings. The DBA/2Lw mice originated from the Heston subline and have been bred in this laboratory for some 20 generations by brother × sister matings. The C57BL/Ka mice were derived from a breeding nucleus supplied by Kaplan and have been bred in this laboratory by brother × sister matings for some 18 generations. A breeding nucleus of the C57L/He mice was obtained directly from Heston 2 years ago. The 4 hybrid groups used were reared in this laboratory from crosses of the above-described inbred strains.

X radiation and bone-marrow treatment.—At 3 to 4 months of age, the mice were exposed to a dose of 800 r X radiation or 900 r for the (C57L × A)F_1 hybrids, which is a 30-day LD100 dose for these animals. Two X-ray tubes placed opposite each other were operated at 200 kvp and 15 ma.; filtration was 0.25 Cu and 0.55 Al with 54 cm. from each focal spot to the center of the mouse, with a dose rate of 120 r per minute. For the irradiation, groups of 10 mice were placed in a lucite box. In order to effect random distribution, the mice were placed in a large cage after irradiation and allowed to intermingle freely before they were divided into experimental groups. At least 3 groups were run concurrently: the irradiated control (noninoculated group), the group receiving F_1 hybrid marrow inoculation, and those receiving parental-strain marrow inoculation. Each cage contained samples from each experimental group.

The bone marrow was prepared as previously described (*1*) with the exception that Locke's solution replaced the buffered saline. The marrow from both femora and both humeri of 1 donor was used for each recipient. All tail-vein inoculations were completed within 3 hours following irradiation. After treatment, the mice were housed in plastic cages with free access to Derwood food pellets and tap water.

Results

Survival.—In considering the survival patterns of these hybrids receiving a lethal total-body dose of X radiation and treated by bone-marrow inoculation, 2 distinct phases will be recognized: 1) the acute phase, which

TABLE 1.—*Effect of isologous or parental-strain bone marrow on survival of (C57BL × A)F_1 hybrids following 800 r total-body X irradiation**

No. mice	Donor marrow	Percent survival at days:			
		14	30	60	90
36	F_1	100	100	96	95
25	C57BL	64	64	35	28
30	A	86	86	50	40
59	None	0	0	0	0

*Data pooled from 8 experiments.

covers the first 14 days post irradiation—the period in which the untreated controls will succumb, and 2) the secondary phase, the period of the late irradiation syndrome. This secondary phase follows the acute phase and varies in duration from a few days to several months. It is marked by the death of animals receiving inoculations of marrow other than isologous marrow.

Table 1 shows the survival pattern of 3 experimental groups of (C57BL × A)F_1 hybrids, each receiving inoculations of hybrid marrow or marrow from either of the parental strains following 800 r total-body X irradiation. In the case of this hybrid, all animals that survived the acute phase of the first 14 days post irradiation survived 30 days. Although the secondary phase (which will be described in more detail later) may have its onset during the first 30-day period, its lethal effects are not evident until the second and third months. Those mice receiving marrow of the parental strains have a marked reduction in the 90-day survival rate as compared with mice inoculated with isologous (hybrid) marrow. The individuality of the parental strains is indicated by a difference between the 2 strains in their ability to protect through the secondary phase.

Table 2 shows the survival pattern of 3 experimental groups of (C57BL × DBA/2)F_1 hybrids each receiving inoculations of hybrid marrow or marrow from either of the parental strains following 800 r total-body irradiation. In this case, the rates of survival at 14 days and 30 days are about the same when hybrid marrow or DBA/2 marrow is used. In the

TABLE 2.—*Effect of isologous or parental-strain bone marrow on survival of (C57BL × DBA/2)F_1 hybrids following 800 r total-body X irradiation**

No. mice	Donor marrow	Percent survival at days:			
		14	30	60	90
81	F_1	87	87	85	81
84	C57BL	82	66	10	7
80	DBA/2	87	81	34	34
115	None	1†	0	0	0

*Data pooled from 17 experiments.
†Died on 15th day.

case of the mice receiving C57BL marrow, however, there is a marked reduction in the 30-day survival over that found at 14 days. In these animals, the onset of the secondary phase is particularly early, starting at 18 to 21 days post irradiation and is emphasized by the greatly reduced 30-day survival. In the case of the mice inoculated with DBA/2 marrow, the onset of the secondary phase is 7 to 10 days later than in those receiving C57BL marrow. The lethal effects of the secondary phase are shown in the reduced survival during the second month post irradiation.

Table 3 shows the survival pattern of 3 experimental groups of (C57L × A)F_1 hybrids, each receiving inoculations of hybrid marrow or marrow from either of the parental strains, following 900 r total-body X irradiation. This survival pattern is quite different from those of the 2 previously described hybrids. The ability of this hybrid to survive the secondary phase, after receiving marrow from the parental strains, is very much greater than in the previously described hybrids, and the lethal effects are spread over a much greater period.

TABLE 3.—*Effect of isologous or parental-strain bone marrow on survival of (C57L×A)F_1 hybirds following 900 r total-body X irradiation**

No. mice	Donor marrow	Percent survival at days:					
		14	30	60	90	120	150
30	F_1	100	100	100	100	100	96
28	C57L	100	100	92	85	78	78
35	A	97	97	88	85	77	77
30	None	3†	0	0	0	0	0

*Data pooled from 8 experiments.
†Died on 15th day.

Table 4 shows the distribution of deaths, over a period of 200 days post irradiation, for (C57L × A)F_1 hybrids given the 3 types of marrow. There is a marked increase in the frequency of deaths after the initial 90-day period in those animals receiving C57L or A marrow. This is in contrast to the situation encountered with 2 other hybrids in which the increased frequency of deaths occurs within the first 90-day period. When the data for animals receiving parental marrow are combined, gastrointestinal hemorrhages accounted for 6 deaths, which occurred 140 to 309 days post irradiation, a frequency lower than that described by Congdon (*9*).

Table 5 gives the survival pattern for irradiated (BALB/c × DBA/2)F_1 hybrids. In the case of this hybrid, however, there is neither a marked difference in survival among the 3 groups receiving the F_1 and parental bone marrows nor is there at any time a marked secondary phase of the irradiation syndrome. The lower survival values here represent deaths from pneumonia primarily. This is not surprising, since the BALB/c strain is particularly susceptible to this disease and so are the hybrids.

Secondary phase of the irradiation syndrome.—In considering the secondary phase at this time, only gross observations will be discussed. Congdon

TABLE 4.—*Distribution of deaths for (C57L × A)F_1 hybrids exposed to 900 r X irradiation and treated with isologous or parental-strain bone marrow*

No. mice	Donor marrow	No. animals dying at 10-day intervals after X ray:																	
		1–30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200
30	F_1											1							
28	C57L		1	1		2				1			1		1	2	1	2	1
35	A		2				1			2		1			1	3	1		
30	None	30																	

TABLE 5.—*Effect of isologous or parental-strain bone marrow on survival of (BALB/c × DBA/2)F_1 hybrids following 800 r total-body X irradiation**

No. mice	Donor marrow	Percent survival at days:			
		14	30	60	90
113	F_1	89	89	88	86
78	BALB/c	88	82	76	70
68	DBA/2	85	82	78	76
127	None	1†	0	0	0

*Data pooled from 19 experiments.
†Two mice died, on 15th and 17th days.

(*9*) has called the secondary phase of the irradiation syndrome the "secondary disease." As a basis for his study, he used (C57L × A)F_1 hybrids as recipients and (101 × C3H)F_1 hybrids as donors. His extensive studies using the (C57L × A)F_1 hybrid as a recipient indicate, at least in gross appearance, a secondary phase of the irradiation syndrome not typical of other hybrids or inbred strains. In testing 6 hybrids, 1 of which was the (C57L × A)F_1 hybrid, and 11 inbred strains, in this laboratory, the typical gross appearance was one of weight loss, resulting from extreme diarrhea, extreme emaciation, and lack of graying of the hair. Death does not necessarily follow rapidly. The animals remain active and may survive for weeks, or even months, in this condition. Liver lesions are occasionally observed but are not of the extreme type described by Congdon. Dermatitis is occasionally seen but is most frequent in strain C57BL/10, and may represent a strain peculiarity. The hemorrhagic lesions in the walls of the gastrointestinal tract, described by Congdon, were found to be absent in all animals except the (C57L × A)F_1 hybrids and represented only a small percentage of the deaths among these hybrids receiving parental-strain marrow. In these experiments the (C57L × A)F_1 hybrids receiving marrow of the parental strain had a brief period of mild diarrhea and weight loss, which could easily have gone unnoticed during the 2nd month post irradiation. Four succumbed during this period. Occasionally, abscesses were observed in the kidneys. These abscesses were most frequently found in (BALB/c × DBA/2)F_1 hybrid males and C57BL/10 males, which are inveterate fighters. Since the abscesses were usually associated with well-chewed tails and scarred hides, they were not considered a necessary manifestation of the secondary phase.

Discussion

It has been demonstrated by Snell (*8*) and others that susceptibility and resistance to tissue transplantation in the mouse are determined by multiple dominant genes called histocompatibility genes. The "strongest" known histocompatibility locus is that designated as *H-2*, of which 10 alleles have been identified. The *H–2* locus not only regulates susceptibility and resistance to tissue transplants but also determines the red-cell antigens. The *H–1* and *H–3* loci are weaker and less fully analyzed (*10*), and their antigenic influence is unknown. With the evidence that

the inoculated marrow does recolonize the irradiated host, the histocompatibility genes, with their effect on the immune mechanism of the donor and the recipient, can no longer be ignored. Considering the genotype at the *H–2* locus of the strains used in these experiments, strain A is *H-2*^{a}; C57BL and C57L are *H-2*^{b}; and BALB/c and DBA/2 are *H-2*^{d}. It becomes obvious immediately that only 1 of these hybrids, namely, (BALB/c × DBA/2)F_1 is homozygous at the *H-2* locus, sharing the same *H-2* allele with both parents. It would, therefore, be antigenically compatible as far as this gene locus is concerned. This is also the only hybrid of those tested in which the F_1 hybrid marrow and either parental-strain marrow afforded equally good protection.

If the immune mechanism of the other 3 hybrids is considered, the F_1 hybrid should not reject the parental-strain marrow, since the hybrid contains all the antigenic components of the parental strain. There is, however, nothing to prevent the parental bone marrow from being immunized against antigens produced by the F_1 hybrid tissues, since the hybrid contains antigenic components from a second parent that may differ from those of the parental strain being tested. The lack of protection through the secondary phase may then be an immune response of the bone-marrow graft against the host. This explanation became more plausible when it was found, as the result of a preliminary experiment, that better protection was afforded by the F_1 hybrid bone marrow in a parental strain than by the bone marrow of the parental strain in the hybrid. The suggestion that a graft reacts against the host has been made by Barnes and Loutit (*11*) and by Billingham *et al.* (*12*). It is unfortunate that the influence of the histocompatibility genes cannot be analyzed in the work of Congdon. In the case of Congdon's work, the hybrid combinations are too complicated for analysis, and, in addition, there is a lack of knowledge of the genetic and antigenic constitution of the strain 101.

Dr. Bernard Amos, of Roswell Park Memorial Institute, using hemagglutination techniques, has detected antibodies that could only be produced by the reaction of the graft against the host in some of the F_1 hybrids treated in this laboratory with parental marrow. This must be considered a preliminary report; more serologic work is necessary to confirm and establish it more completely.

Summary

When F_1 hybrid mice are exposed to a lethal dose of X radiation and treated with bone marrow, the F_1 marrow affords better protection than either of the parental strains. This phenomenon is called the "F_1 hybrid effect."

The results of the inoculation of parental marrow are similar to those found when homologous marrow is used. Death occurs during the secondary phase of the irradiation syndrome and is marked particularly by extreme diarrhea and emaciation.

There is a positive correlation between the histocompatibility gene locus, *H-2*, and the ability to protect the recipient during the secondary phase.

Evidence at present indicates that an immune response of the graft against the host, when the graft and host have different histocompatibility genotypes at the *H-2* locus, is responsible for the secondary phase of the irradiation syndrome.

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ERRATUM

Volume 18, Number 5, May 1957
Journal of the National Cancer Institute

In the paper entitled "Effect of Prolonged Administration of Spermicidal Contraceptives on Rats Kept on Low-Protein or on Full Diet," by Hoch-Ligeti:

PAGE 668, LAST PARAGRAPH, and PAGE 669, SUMMARY—FIFTH PARAGRAPH: *for* "8-*ortho*-hydroxyquinoline" *read* "8-hydroxyquinoline."

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